Expression of a cDNA for the catalytic subunit of skeletal-muscle phosphorylase kinase in transfected 3T3 cells

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Phosphorylase kinase is a multimeric enzyme of composition $(\alpha, \beta, \gamma, \delta)_4$ whose catalytic activity resides in the γ -subunit. As an approach to understand further its regulation, a cDNA for the γ -subunit of phosphorylase kinase (γ PhK) has been cloned into a mammalian expression vector behind the mouse metallothionein-1 promoter. NIH 3T3 cells were co-transfected with this construct (pEV γ PhK) and pSV2neo, G418-resistant clones were selected, and several were found to have stably incorporated the γ -subunit cDNA into their genomic DNA. Phosphorylase kinase activity was clearly present in extracts from cultures of pEV γ PhK-transformed cells and increased several-fold after 24 h of incubation with Zn^{2+} , whereas it was undetectable in the parent 3T3 cells. A significant, but variable, proportion (15–70 %) of the activity was Ca^{2+} -dependent. We conclude that the phosphorylase kinase activity expressed by the cells transformed with pEV γ PhK is due to free γ -subunit and γ -subunit associated with cellular calmodulin, which replaces the δ -subunit normally associated with the γ -subunit in the holoenzyme.

INTRODUCTION

Phosphorylase kinase, a large multimeric enzyme of subunit composition $(\alpha, \beta, \gamma, \delta)_4$, is a key enzyme in the physiological control of glycogen metabolism. It is regulated in response to at least two second-messenger systems, cyclic AMP (via the cyclic AMP-dependent protein kinase) and Ca^{2+} , as well as by several other allosteric modulators (e.g. ADP and glycogen). Current knowledge of its structure and control has recently been reviewed (Pickett-Gies & Walsh, 1986).

The catalytic activity of the enzyme resides with the γ -subunit (Reimann *et al.*, 1984; Chan & Graves, 1982*a*) and is regulated by the other subunits. The α - and β -subunits appear to act by suppressing the activity of the γ -subunit, and phosphorylation of α - and β -subunits releases that inhibition (Chan & Graves, 1982*a,b*). The inhibition can also be released by limited proteolysis of the α - and β -subunits, or by increasing the pH. The strong Ca^{2+} -dependence of the enzyme is regulated via the δ -subunit, which is essentially identical with calmodulin, but is an integral part of the holoenzyme and associated with the γ -subunit even in the absence of Ca^{2+} (Cohen *et al.*, 1978; Grand *et al.*, 1981). The isolated $\gamma\delta$ complex derived from the holoenzyme also exhibits Ca^{2+} -stimulated phosphorylase kinase activity (Chan & Graves, 1982*c*), although its dependence on Ca^{2+} is less than that of the holoenzyme. Ca^{2+} -stimulation of the skeletal-muscle holoenzyme also occurs as a consequence of a Ca^{2+} -dependent binding of exogenous calmodulin to the α - and β -subunits (DePaoli-Roach *et al.*, 1979; Walsh *et al.*, 1980).

Our understanding of the interaction between the subunits of phosphorylase kinase has been limited by the difficulty of separating the subunits in a way which preserves their functional integrity. The conditions required to separate the α -, β - and δ -subunits from the γ -subunit are quite harsh (Chan & Graves, 1982*b*; Kee &

Graves, 1986; Paudel & Carlson, 1987), and the subunit and subunit complexes thus isolated ($\alpha\gamma\delta$, $\gamma\delta$ and γ) may not fully reflect the behaviour of native subunits. In particular, isolated active γ -subunit has thus far only been obtained after its renaturation (Kee & Graves, 1986; Paudel & Carlson, 1987). Undenatured isolated α - and β -subunits have not yet been obtained. An alternative approach for the study of the enzyme is to express the subunits of phosphorylase kinase in a system in which the other subunits are not available. In the present paper we describe the stable transformation of 3T3 cells with a cDNA for the γ -subunit of phosphorylase kinase, and the expression of phosphorylase kinase catalytic activity in these cells.

MATERIALS AND METHODS

Construction of phosphorylase kinase γ -subunit expression vector (pEV γ PhK)

A cDNA for the γ -subunit of phosphorylase kinase (γ PhK) was obtained from a rat soleus-muscle library in λ gt10 (Cawley *et al.*, 1988), and the 1.4 kb restriction-*Eco*R1 insert containing the complete coding region for the γ -subunit was subcloned into pUC18. The unique *Bam*H1 site in the insert is located 20 nucleotides upstream from the ATG initiation codon; the *Bam*H1 insert from this construct contains the entire coding region, plus 20 nucleotides of 5' untranslated cDNA, 128 nucleotides of 3' untranslated cDNA, and 30 nucleotides from the pUC polylinker on the 3' end. This insert was cloned into the *Bgl*II site of the Zem3 expression vector (a gift from Dr. G. Stanley McKnight) (Uhler & McKnight, 1987). Constructs were restriction-mapped and partially sequenced to verify correct orientation of the coding region between the mouse metallothionein-1 (MT-1) promoter and the human-growth-hormone poly(A) addition region of the Zem3 vector.

Abbreviations used: γ PhK, γ -subunit of phosphorylase kinase; MT-1, metallothionein-1.

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Transfection and selection of clones containing γ PhK cDNA

Mouse NIH 3T3 fibroblasts were grown in Dulbecco's modified Eagle medium supplemented with 10% (v/v) newborn-calf serum, and passaged 24 h before transfection, to 2×10^5 cells per 10 cm plate. A 10 cm plate was transfected with 18 μ g of supercoiled γ PhK expression vector (pEV γ PhK) and 2 μ g of the pSV2neo plasmid, which encodes a gene conferring resistance to the neomycin-kanamycin antibiotics (including G418, which blocks protein synthesis in eukaryotic cells; Southern & Berg, 1982). A modified $\text{Ca}_3(\text{PO}_4)_2$ procedure (Chen & Okayama, 1987) was used. After overnight incubation with the $\text{Ca}_3(\text{PO}_4)_2$ -DNA precipitate, the cells were washed twice and refed with complete medium. The following day they were subcultured to 4×10^5 cells/10 cm dish (approx. 1:4), and 24 h later G418 (Geneticin; Gibco, New York, NY, U.S.A.) was added to a final concentration of 40 μ g/ml. The cells were maintained in the selective medium (fed every 4 days) for 3 weeks, at which time G418-resistant colonies were cloned using glass cylinders and grown for further characterization. Stable G418-resistant lines were maintained in Dulbecco's modified Eagle medium containing 10% supplemented bovine calf serum (Hyclone, Logan, UT, U.S.A.) and G418 (200 μ g/ml). For induction of the MT-1 promoter, ZnSO_4 was added to the culture medium from 10 mM stock at the concentrations and times indicated in the Figure legends.

Genomic DNA prepared from confluent cultures of G418-resistant clones and parental 3T3 cells (Strauss, 1987) was denatured in 0.3 M-NaOH, diluted in 10 vol. of 3 M-NaCl/0.3 M-sodium citrate, pH 7.0, and applied to nitrocellulose (0.1–10 μ g/spot), using a 96-well vacuum manifold (Bethesda Research Laboratories). Denatured pEV γ PhK DNA (0.1–100 pg) was dotted alongside as a standard. The dot blots were baked for 2 h under vacuum, prehybridized in hybridizing buffer [0.75 M-NaCl/50 mM- NaH_2PO_4 (pH 7.4)/5 mM-EDTA/0.1% bovine serum albumin/0.1% Ficoll/0.1% polyvinylpyrrolidone/0.1% SDS/salmon sperm DNA (0.1 mg/ml)/50% formamide] and hybridized in the same buffer overnight at 42 °C. The cDNA probe (1.4 kb insert from the pUC18 construct) was labelled with ^{32}P by Klenow extension of random primers. The blots were washed at 44 °C in 0.1% SDS/30 mM-NaCl/3 mM-sodium citrate, pH 7.0.

Phosphorylase kinase assay

Cells were harvested by trypsin treatment, washed once in medium containing 10% serum and twice in PBS, then resuspended in Extraction Buffer [30 mM-Tris (pH 7.5)/30 mM-KCl/0.05% Triton X-100/45 mM-2-mercaptoethanol/aprotinin (20 munits/ml) (or 2 mM-benzamidine)/1 mM-phenylmethylsulphonyl fluoride and 0.1 mM-tosyl-L-phenylalanylchloromethane], usually to $1\text{--}2 \times 10^6$ cells/ml. After homogenization (ten strokes with a glass/Teflon homogenizer), aliquots were removed from DNA determination, and the remaining extract was centrifuged for 2 min in an Eppendorf microcentrifuge (model 5415). These supernatants were used directly in the assay. Pure rabbit skeletal-muscle phosphorylase kinase, prepared as described by Pickett-Gies & Walsh (1985), was diluted in extraction buffer before the assay; the enzyme activity in this buffer was indistinguishable from the activity of the enzyme diluted in the standard

dilution buffer (10 mM- β -glycerol phosphate/45 mM-2-mercaptoethanol). The assay of phosphorylase kinase was performed as described by Angelos *et al.* (1987), except that the final concentration of CaCl_2 in the assay was 0.1 mM, the final pH was either 6.8 or 8.2 and the incubation was typically 40 min. Background activities (no extract or enzyme added) have been subtracted from all of the data presented. For each assay, multiple (two to six) plates of cells, from the same set of growth conditions and Zn^{2+} stimulation, were pooled, and the assay performed in duplicate or triplicate. Within an assay, duplicates or triplicates were within 10%. When activities were determined relative to cell number, cellular protein and cellular DNA, each method gave consistent conclusions. Phosphorylase *b* was prepared as detailed by Sul *et al.* (1983), and used at a final concentration of 5 mg/ml. Where substituted as a potential substrate, histone type IIA (Sigma Chemical Co.) was used at a final concentration of 2.5 mg/ml. Protein determination was by the method of Bradford (1976). DNA in cell extracts was quantified by its binding to the Hoechst compound H33258 (Labarca & Paigen, 1980).

RNA purification and analysis

For preparation of RNA, cells were harvested and washed as for cell extracts, then resuspended at approx. 10^7 cells/ml in lysis buffer [0.14 M-NaCl/1.5 mM-MgCl/10 mM-Tris (pH 8.6)/0.5% Nonidet P-40]. Lysates were underlaid with an equal volume of lysis buffer plus 20% (w/v) sucrose and centrifuged at 2000 g for 10 min at 25 °C. The upper layer was recovered, added to an equal volume of 0.2 M-Tris (pH 7.0)/25 mM-EDTA/0.3 M-NaCl/2% SDS, and incubated with proteinase K (200 μ g/ml) at 37 °C for 45 min. The RNA was extracted twice with phenol/chloroform (1:1, v/v) and once with chloroform, and the RNA precipitated in ethanol. Recoveries were approximately 100 μ g/ 10^7 cells.

For Northern-blot analysis, samples of total RNA were denatured in glyoxal/dimethyl sulphoxide (Selden, 1987) and separated in a 1%-agarose gel in 10 mM- Na_2HPO_4 , pH 7.0. The lanes containing molecular-mass-standards were stained with ethidium bromide, whereas the lanes containing the cellular RNA were blotted to nitrocellulose in 3 M-NaCl/0.3 M-sodium citrate. The blots were baked and hybridized with the [^{32}P]cDNA probe as described above for DNA blots.

RESULTS AND DISCUSSION

A plasmid which would direct the expression of γ PhK in mammalian cells was constructed by using a cDNA for this protein isolated previously from rat soleus muscle (Cawley *et al.*, 1988). To construct pEV γ PhK (Fig. 1), the cDNA was cloned into the expression vector Zem3 (Uhler & McKnight, 1987). In this construct the mouse MT-1 promoter directs initiation of transcription approx. 80 nucleotides upstream from the ATG of the γ PhK cDNA. The 3' flanking region of the human-growth-hormone gene, located approx. 60 nucleotides beyond the 3' flanking region of the cDNA, should ensure polyadenylation of the transcript.

NIH 3T3 cells were co-transfected with pEV γ PhK and pSV2neo, and G418-resistant clones were selected. The number of copies of γ PhK per genome relative to the parent 3T3 cell line was determined by dot-blot analysis of genomic DNA (Fig. 2). Two of the clones, γ 3 and γ 20,

Fig. 1. Structure of the γ PhK expression vector pEV γ PhK

The vector was constructed as described in the Materials and methods section. Within the boxed area, which indicates the insert which was cloned into the Zem3 vector, the stippled area represents the coding region for the γ -subunit of phosphorylase kinase and the open areas represent non-coding regions of the γ PhK cDNA. The hatched region represents the small part of the polylinker from the pUC18 construct. The heavy lines indicate the positions of the mouse MT-1 promoter (with the transcription start site indicated at +1) and the human-growth-hormone (hGH) polyadenylation regions of the vector. The direction of transcription of the γ PhK cDNA and the β -lactamase gene are indicated by arrows. Also denoted are the ATG initiation codon and Amp^R, the β -lactamase gene conferring ampicillin resistance.

Fig. 2. DNA dot-blot analysis of several G418-resistant clones obtained after transformation of 3T3 cells with pEV γ PhK

Genomic DNA prepared from several G418-resistant clones (0.1–1 μ g, a), and plasmid DNAs (pEV γ PhK and Zem3; 0.01–100 pg each, b) were dotted on to nitrocellulose as described in the Materials and methods section. The blots were probed with [³²P]cDNA (10⁵ c.p.m./ml). No sample was applied for γ 20 at 1 μ g of genomic DNA or for γ 11, γ 12, γ 13 or γ 16 below 1 μ g.

Fig. 3. Northern-blot analysis of γ -subunit mRNAs in 3T3 cells and cells expressing pEV γ PhK

Cultures of 3T3, γ 3 and γ 20 cells were treated with 0, 70 or 90 μ M-ZnSO₄ for 18 h. The cells were harvested and washed, and aliquots removed for phosphorylase kinase assay. Total RNA was extracted from the remaining cells, and 20 μ g of each was denatured, electrophoresed through agarose, blotted to nitrocellulose and probed with [³²P]cDNA for γ PhK as described in the Materials and methods section. In the control lane designated 'AH', 20 μ g of RNA from the γ 20 cells treated with 90 μ M-Zn²⁺ were subjected to alkaline hydrolysis (0.3 M-NaOH; 20 min at 68 °C) and neutralized before denaturation in glyoxal/dimethyl sulphoxide, to demonstrate that the signals seen were due to RNA. The results of the phosphorylase kinase assay are shown directly below each lane. Note: this Figure represents two separate experiments, namely one in which RNAs from the wild-type 3T3, γ 3 and γ 20 cell lines were prepared (Expt. 1), and a separate experiment in which only γ 3 cells from a different culture were examined (Expt. 2). The phosphorylase kinase activity (PhKA, in pmol of ³²P/min per 10⁶ cells) data were obtained from extracts prepared from frozen cell pellets of each group, all assayed at the same time.

Fig. 4. Induction of phosphorylase kinase activity in cells expressing pEV γ PhK as a function of Zn²⁺ concentration

Cell extracts were prepared from near-confluent cultures of pEV γ PhK-expressing clones γ 3 (hatched bars) and γ 20 (open bars), which were treated with the indicated concentrations of ZnSO₄ for 18 h before harvest. Phosphorylase kinase was assayed as described in the Materials and methods section.

Fig. 5. Reaction progress curves for phosphorylase kinase from cells expressing pEV γ PhK

Cell extracts were assayed for phosphorylase kinase activity as described in the Materials and methods section, except that $30 \mu\text{l}$ of extract was added to $150 \mu\text{l}$ of reaction mix, and $40 \mu\text{l}$ aliquots were removed at the times indicated. Extracts were from 3T3 cells ($\square$, $\blacksquare$) and from pEV γ PhK-expressing clones $\gamma 3$ ($\triangle$, $\blacktriangle$) and $\gamma 20$ ($\circ$, $\bullet$). Filled symbols indicate that the cells were treated with $90 \mu\text{M}$ ZnSO_4 for 24 h before harvest. (a) Complete reaction mixture in the presence of phosphorylase *b*; (b) control data in the absence of phosphorylase *b*. Cell extract protein concentrations in the reaction mixtures were approx. 0.1 mg/ml .

were selected for further study on the basis of their high copy numbers of γ PhK cDNA, which were estimated at 500 and 1000 respectively.

RNAs were prepared from near-confluent cultures of 3T3, $\gamma 3$ and $\gamma 20$ cells, either without or with 18 h of incubation with ZnSO_4 (70 or $90 \mu\text{M}$), and subjected to Northern-blot analysis (Fig. 3). The major species of RNA which hybridized to the γ PhK cDNA probe is approx 1.8 kb long, consistent with the expression of the 1.4 kb cDNA plus additional 3' untranslated and poly(A) sequence. This band was not detectable in RNAs from the wild-type 3T3 cells or from $\gamma 3$ cells without Zn^{2+} treatment, but incubation of the $\gamma 3$ cells with Zn^{2+} resulted in substantial induction of this species. In untreated $\gamma 20$ cells, low, but detectable, amounts of this RNA were found, and the abundance of this species was markedly increased as a function of Zn^{2+} concentration.

Cell extracts made from cultures treated with various concentrations of Zn^{2+} were assayed for phosphorylase kinase activity, and the results are shown in Fig. 4. The enzyme activity (whether expressed in pmol of $^{32}\text{P}/\text{min}$ per cell number as shown, or per μg of DNA or protein)

increased as a function of Zn^{2+} concentration. At higher concentrations, however, the Zn^{2+} was increasingly toxic to the cells (results not shown). Good induction of enzyme activity with modest cell death was usually achieved in near-confluent cells with $90 \mu\text{M}$ ZnSO_4 , and this concentration was chosen as the standard induction dose for subsequent experiments.

In order to characterize further the phosphorylase kinase activity expressed by the cells containing the γ PhK cDNA, cell extracts were made from near-confluent cultures of 3T3, $\gamma 3$ and $\gamma 20$ cells. Fig. 5 shows the time course of the phosphorylase kinase assay for these extracts. The assay was close to linear for at least 40 min, which is the time point used in all subsequent assays. As shown in Fig. 5 and also in Table 1, which presents the characteristics of the expressed activity in several sets of experiments, the parent 3T3 cells had no detectable phosphorylase kinase activity, with or without Zn^{2+} addition. The $\gamma 20$ extracts, and in most cases the $\gamma 3$ cells, had some phosphorylase kinase activity, even without Zn^{2+} addition to the cultures. This uninduced activity was consistently greater for $\gamma 20$ cells than for $\gamma 3$, regardless of whether the data were normalized to cell number (Fig. 5 and Table 1), cellular protein or DNA (results not shown); this is likely due to the larger number of copies of the cDNA per genome in the $\gamma 20$ cells. Phosphorylase kinase activity was induced as a consequence of Zn^{2+} treatment. The levels of induction obtained under standard induction conditions ($90 \mu\text{M}$ ZnSO_4 , 24 h) varied typically from 3- to 15-fold for $\gamma 3$ and from 1.5- to 7-fold for $\gamma 20$, and depended in part on the growth conditions of the cells. Under similar growth conditions the fold induction of the $\gamma 3$ cells by Zn^{2+} was consistently greater than that of the $\gamma 20$ cells, but the total level of activity was generally higher in the $\gamma 20$ cells. On the basis of the established activity of purified phosphorylase kinase at pH 8.2 and that of reactivated γ -subunit, and assuming no interference from other constituents of cell extract, an activity at pH 8.2 of 1500 pmol of $^{32}\text{P}/\text{min}$ per 10^6 cells would correspond to the synthesis of approx. 40 ng of active enzyme protein per 10^6 cells, representing about 0.007% of total cellular protein in the Zn^{2+} -induced $\gamma 20$ cells.

The enzyme activity measured in $\gamma 3$ and $\gamma 20$ extracts was fully dependent upon the presence of phosphorylase *b* as substrate (Fig. 5b). Because there are no other enzymes known to phosphorylate phosphorylase *b* (Krebs, 1986; Madsen, 1986), this argues that the activity observed in the cell extracts was due to the expressed γ PhK, as opposed to other kinases in the cells which might phosphorylate endogenous substrates in the extracts. The specificity of the enzyme activity in the extract was further explored by using histone as a substrate (Table 1). (Histone is negligibly phosphorylated by purified phosphorylase kinase, at a rate less than 1% of that observed for phosphorylase *b*). The cell extracts did exhibit some endogenous histone kinase activity, but it was no greater in the two clones expressing pEV γ PhK than it was in wild-type 3T3 cells and was not responsive to Zn^{2+} induction. We therefore conclude that this histone kinase activity is not relevant to the expression of pEV γ PhK, but instead is due to the activity of other protein kinases present in normal T3 cell extracts.

The variability observed between experiments in the amount of expressed phosphorylase kinase activity in the pEV γ PhK-transfected cells (see in particular Table 1) is

Table 1. Activity of phosphorylase kinase and histone kinase from pEV γ PhK-expressing cells

Cell extracts were prepared from 3T3 cells and from cells expressing pEV γ PhK (γ 3 and γ 20) either with or without Zn²⁺ induction (denoted by + or -). The data are from four separate experiments (1-4) in which different sets of extracts were assayed. Protein kinase activity was measured with either phosphorylase (5 mg/ml) or histone type IIA, (2.5 mg/ml) at either pH 6.8 or 8.2, either without or with 0.4 mM-EGTA, as specified. All activities are expressed in pmol of ³²P/min per 10⁶ cells.

Expt. no.	Substrate...		Phosphorylase <i>b</i>					Histone	
	Clone/ Zn ²⁺ induced or not	Activity ratio (pH 6.8/ 8.2)	Activity at:		Fold induction by Zn ²⁺ (pH 8.2)	Activity at pH 8.2 + EGTA	Fraction EGTA- insensitive	Activity at:	
			pH 6.8	pH 8.2				pH 6.8	pH 8.2
1	3T3/-		9	9		4		117	99
	3T3/+		27	18		22		157	135
3	3T3/-		9	18		9		85	108
	3T3/+		18	31		27		103	148
4	3T3/-		40	58		36		225	306
	3T3/+		31	27		40		193	279
1	γ 3/-	0.64	40	63		31	0.50	135	108
	γ 3/+	0.63	301	472	7.5	288	0.60	103	112
2	γ 3/-	0.50	45	90		31	0.35	117	144
	γ 3/+	0.42	315	751	8.3	639	0.85	171	211
3	γ 3/-	0.50	49	99		49	0.50	103	171
	γ 3/+	0.30	144	477	4.8	279	0.58	198	288
2	γ 20/-	0.44	171	387		135	0.34	85	108
	γ 20/+	0.40	585	1453	3.7	1192	0.82	153	166
3	γ 20/-	0.37	135	364		112	0.30	117	103
	γ 20/+	0.25	157	612	1.7	306	0.50	126	198
4	γ 20/-	0.30	409	1053		373	0.35	238	409
	γ 20/+	0.47	1498	3172	3.0	2461	0.77	265	396

Fig. 6. Effect of EGTA on the activity of phosphorylase kinase from extracts of cells expressing γ PhK cDNA

Phosphorylase kinase (holoenzyme) ($\square$) and cell extracts were assayed at pH 8.2 as described in the Materials and methods section, except that, in addition to 0.1 mM-CaCl₂, the reaction mixtures contained EGTA at the indicated concentrations. Cells of clones γ 3 (Δ , $\blacktriangle$) and γ 20 ($\circ$, $\bullet$) were grown to near-confluence, and some plates of each set were treated with 90 μ M-ZnSO₄ for 24 h before harvest (filled symbols).

at least partly determined at the level of transcription. In Fig. 3, for example, the RNAs from two separate experiments using γ 3 clones were examined on the same Northern blot. The relative abundance of γ PhK mRNA from the Zn²⁺-induced cultures was quite different for the two experiments, as were the phosphorylase kinase activities expressed by the cells (shown below the corresponding RNA lanes). It should be noted that, in the experiments in Fig. 3, there was a consistent pattern in all sets of cells, that at the higher Zn²⁺ concentration the higher levels of transcript were associated with higher levels of expressed enzyme activity. However, there was not a strict proportionality between the relative abundance of mRNA and enzyme activity, suggesting that the expression and/or stabilization of enzymically active produce may be complex.

The phosphorylase kinase activity expressed by the cells containing pEV γ PhK was examined in the presence of EGTA; several results from this set of experiments are of interest. The activity was inhibited by EGTA (Fig. 6; see also Table 1), but a significant and variable fraction (30-85 %) of the activity from cells expressing pEV γ PhK was Ca²⁺-independent. This is in contrast with phosphorylase kinase holoenzyme, which is nearly totally inhibited by EGTA (> 95 %; Fig. 6; Pickett-Gies & Walsh, 1986). Interestingly, the Ca²⁺-independent fraction was consistently higher in Zn²⁺-induced cells than in the corresponding untreated cells. For all extracts in which the effect of 0.4 mM-EGTA has been tested, the fraction of EGTA-insensitive activity was 0.50 ± 0.18 (mean $\pm$ s.d., $n = 13$) for γ 3 compared with 0.61 ± 0.12 ($n = 14$) for Zn²⁺-induced γ 3, and 0.39 ± 0.12 ($n = 14$) for Zn²⁺-induced γ 20.

for $\gamma 20$, compared with 0.69 ± 0.15 ($n = 15$) for Zn^{2+} -induced $\gamma 20$. [In the assay itself, neither enzyme activity nor EGTA inhibition of activity was affected by Zn^{2+} at concentrations up to $60 \mu\text{M}$ (results not shown); the intracellular concentration of Zn^{2+} would have needed to exceed 4 mM to give $60 \mu\text{M}$ in the assay mixture.] The isolated γ -subunit (obtained by renaturation after h.p.l.c. separation of the holoenzyme) has been reported to be totally Ca^{2+} -independent (Kee & Graves, 1986). The $\gamma\delta$ complex, isolated from the holoenzyme by LiBr dissociation, is EGTA-sensitive, but not nearly to the same extent as is observed with the holoenzyme, since EGTA-insensitive activity constitutes 30–40% of the total for the $\gamma\delta$ complex (at pH 8.2) (Chan & Graves, 1982a). The EGTA-sensitivity that we have observed in extracts prepared from pEV γ PhK-expressing cells, and the total activity, have been, as noted above, quite variable. These data are compatible with the extracts containing a mixture of free γ -subunit and γ -subunit associated with cellular calmodulin, but in variable proportions. Consistent with the presence of some free subunit in the extract, calmodulin addition was found to cause an increase in total activity (from 1.1 to 1.7-fold) and a concomitant increase in Ca^{2+} -dependency (results not shown); this would presumably be due to the association of the free γ -subunit with the added exogenous calmodulin. The relative amounts of γ -subunit and γ -subunit-calmodulin complex in cell extracts expressing pEV γ PhK are likely to depend on a variety of conditions. For example, the greater EGTA-insensitivity for enzyme from Zn^{2+} -induced cultures is likely due to a greater proportion of the enzyme present as free γ -subunit (compared with γ -subunit associated with calmodulin) than in uninduced cultures. Conditions which may affect the amount of Ca^{2+} -dependent (i.e. calmodulin-associated) γ -subunit would induce the relative availability of cellular calmodulin and whether this might be modulated by the presence of expressed γ PhK.

The ratio of activities at pH 6.8 and 8.2 for phosphorylase kinase, which is commonly used in characterization of the enzyme, was somewhat variable in the $\gamma 3$ and $\gamma 20$ cells expressing pEV γ PhK, as shown in Table 1. The average of the pH 6.8/8.2 activity ratio in all assays done to date with the transfected cells was 0.49 (S.D. = 0.13; $n = 22$), with a range of 0.25–0.68. No differences were readily apparent between the $\gamma 3$ and $\gamma 20$ clones either with or without Zn^{2+} induction. For non-activated multimeric phosphorylase kinase this ratio is typically ~ 0.05 ; activation, as by phosphorylation of the α - and β -subunits, increases the ratio, typically to 0.2–0.6, presumably because the inhibitory effect of the α - and β -subunits upon the γ -subunit is released. This suggests that the activity observed in the pEV γ PhK-expressing clones is due to γ -subunit, which is uninhibited by the α - and β -subunits, either because they are not present in significant quantities, which is most likely, or because these subunits, if associated with the γ -subunit are in an activated state (e.g. phosphorylated). The $\gamma\delta$ complex, which has been isolated after dissociation of phosphorylase kinase with 1.8 M-LiBr (Chan & Graves, 1982a,c) has a reported pH activity ratio of near unity. The free γ -subunit, renatured after h.p.l.c. separation of the holoenzyme (Kee & Graves, 1986), is sensitive to pH, with a pH 6.8/8.2 activity ratio of 0.5–0.6. For phosphorylase kinase activity in extracts from pEV γ PhK-expressing cells, the observed variability of

the pH activity ratio may be due to differing proportions of free γ -subunit and γ -subunit-calmodulin in the extracts, the presence of both of which was also suggested from the studies of Ca^{2+} -sensitivity described above. A direct correlation between pH activity ratio and EGTA-sensitivity was not readily apparent for the cell extracts but, as has been previously described (Pickett-Gies *et al.*, 1987), the pH activity ratio of phosphorylase kinase in crude tissue extracts can be compromised by other cellular constituents. In some cases the pH activity ratio observed for the extracts from transfected 3T3 cells was lower than one would expect from either the γ -subunit or the $\gamma\delta$ complex, isolated biochemically. It is possible that the ratios previously reported for isolated γ -subunit and/or $\gamma\delta$ complex may have been high owing to damage incurred during the harsh conditions needed for their isolation.

It appears likely that this system for the expression of the catalytic subunit of phosphorylase kinase in 3T3 cells will allow further resolution of the control of the activity of this important and highly regulated enzyme. We are particularly interested in exploring the interactions between the γ -subunit and the other subunits of phosphorylase kinase, using biochemically isolated or expressed cDNAs for the α - and β -subunits. In addition, alterations of the protein structure of the γ -subunit will be important for elucidating many aspects of the catalytic activity, regulation and subunit interactions of this enzyme.

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REFERENCES

- Angelos, K. L., Ramachandran, C. & Walsh, D. A. (1987) *J. Biol. Chem.* **262**, 3219–3226
- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- Cawley, K. C., Ramachandran, C., Gorin, F. A. & Walsh, D. A. (1988) *Nucleic Acids Res.* **16**, 2355–2356
- Chan, K.-F. J. & Graves, D. J. (1982a) *J. Biol. Chem.* **257**, 5948–5955
- Chan, K.-F. J. & Graves, D. J. (1982b) *J. Biol. Chem.* **257**, 5939–5947
- Chan, K.-F. J. & Graves, D. J. (1982c) *J. Biol. Chem.* **257**, 5956–5961
- Chen, C. & Okayama, H. (1987) *Mol. Cell. Biol.* **7**, 2745–2752
- Cohen, P., Burchell, A., Foulkes, J. G., Cohen, P. T. W., Vanaman, T. C. & Nairn, A. C. (1978) *FEBS Lett.* **92**, 287–293
- DePaoli-Roach, A. A., Gibbs, J. B. & Roach, P. J. (1979) *FEBS Lett.* **105**, 321–328
- Grand, R. J. A., Shenolikar, S. & Cohen, P. (1981) *Eur. J. Biochem.* **113**, 359–369
- Kee, S. M. & Graves, D. J. (1986) *J. Biol. Chem.* **262**, 9448–9453
- Krebs, E. G. (1986) *Enzymes* 3rd Ed. **17**, 3–20
- Labarca, C. & Paigen, K. (1980) *Anal. Biochem.* **102**, 344–352
- Madsen, N. B. (1986) *Enzymes* 3rd Ed. **17**, 365–394
- Paudel, H. K. & Carlson, G. M. (1987) *J. Biol. Chem.* **262**, 11912–11915
- Pickett-Gies, C. A. & Walsh, D. A. (1985) *J. Biol. Chem.* **260**, 2046–2056

- Pickett-Gies, C. A. & Walsh, D. A. (1986) *Enzymes* 3rd Ed. **17**, 395–459
- Pickett-Gies, C. A., Carlsen, R. C., Anderson, L. J., Angelos, K. L. & Walsh, D. A. (1987) *J. Biol. Chem.* **262**, 3227–3238
- Reimann, E. M., Titani, K., Ericsson, L. H., Wade, R. D., Fischer, E. H. & Walsh, K. A. (1984) *Biochemistry* **23**, 4185–4192
- Selden, R. F. (1987) in *Current Protocols in Molecular Biology* (Ausbel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. D., Smith, J. A. & Struhl, K., eds.), pp. 4·9·5–4·9·8, John Wiley and Sons, New York
- Southern, P. J. & Berg, P. (1982) *J. Mol. Appl. Genet.* **1**, 327–341
- Strauss, W. M. (1987) in *Current Protocols in Molecular Biology* (Ausbel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. D., Smith, J. A. & Struhl, K., eds.), pp. 2·2·1–2·2·3, John Wiley and Sons, New York
- Sul, H. S., Dirden, B., Angelos, K. L., Hallenbeck, P. C. & Walsh, D. A. (1983) *Methods Enzymol.* **99**, 250–259
- Uhler, M. D. & McKnight, G. S. (1987) *J. Biol. Chem.* **262**, 15202–15207
- Walsh, K. X., Millikin, D. M., Schlender, K. K. & Reimann, E. M. (1980) *J. Biol. Chem.* **255**, 5036–5042

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