The Testis Isoform of the Phosphorylase Kinase Catalytic Subunit (PhK-γT) Plays a Critical Role in Regulation of Glycogen Mobilization in Developing Lung*

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In order to identify the form of phosphorylase kinase catalytic subunit expressed in developing lung, degenerate polymerase chain reaction primers were designed based on conserved domains of the two known catalytic subunits, expressed primarily in muscle and testis. Amplification of cDNA from day 19 fetal rat lung followed by cloning and sequence analyses indicated that only the testis isoform of phosphorylase kinase (PhK-γT) was detectable in fetal lung. In situ hybridization analyses indicated that expression of PhK-γT RNA in developing lung tissue was widespread and not restricted to Type II epithelial cells; PhK-γT protein expression was temporally and spatially correlated with expression of PhK-γT RNA. PhK-γT RNA and protein expression was also characterized in the PhK-deficient glycogen storage disease (gsd) rat. PhK-γT RNA levels were similar in Type II cells isolated from wild type and gsd/gsd fetuses; in contrast, PhK-γT protein was virtually undetectable in gsd/gsd Type II cells and enzyme activity was very low. These results suggest that PhK-γT plays a critical role in mobilization of glycogen during fetal lung development and that failure to catabolize glycogen in the gsd/gsd rat is related to an untranslatable PhK-γT RNA or unstable protein.

The epithelium of the fetal respiratory tree is characterized by the accumulation of glycogen during the last part of gestation. Glycogen mobilization is both temporally and spatially regulated such that catabolism in the proximal airways precedes at or close to birth, whereas catabolism in the distal airway occurs earlier coinciding with maturation of the Type II epithelial cell (1). On day 17 of gestation, the respiratory epithelium of the fetal rat lung consists of undifferentiated columnar cells that contain small amounts of glycogen; lamellar bodies, the intracellular storage granules for surfactant, are not detectable (2). On day 19 of gestation, the epithelium consists of cuboidal cells which contain large pools of glycogen and a few lamellar bodies. By day 21 of gestation, the epithelium approximates that observed in postnatal life consisting of thin Type I cells and cuboidal Type II cells which are devoid of glycogen and contain numerous lamellar bodies. Kikkawa and co-workers (3) noted the inverse relationship between glycogen and lamellar bodies and speculated that glycogenolysis might be associated with surfactant synthesis. A direct link between the breakdown of glycogen on day 20 of gestation and surfactant synthesis was demonstrated by Farrell and Bourbon (4) who showed that glucose, originating from glycogen, provided substrate for fatty acid and glycerol synthesis which, in turn, were incorporated into surfactant phosphatidylcholine. Both biochemical and morphological studies therefore support the hypothesis that glycogen breakdown may be an important event in perinatal surfactant phospholipid synthesis.

The initial step in glycogen catabolism is the phosphorylolyis of glycogen to glucose 1-phosphate by phosphorylase a, the activated form of glycogen phosphorylase. Glycogen phosphorylase enzyme activity has been shown to increase in late gestation fetal lung concomitant with initiation of glycogen degradation (5). In contrast to the developmentally regulated increase in enzyme activity, the level of RNA encoding brain glycogen phosphorylase, the predominant isoform of glycogen phosphorylase, is elevated in gsd/gsd fetuses leading to an accumulation of the inactive form glycogen phosphorylase and failure to mobilize pulmonary glycogen, ultimately resulting in reduced phosphatidylcholine synthesis (8). These results suggest that post-translational phosphorylation of glycogen phosphorylase by PhK is a critical step in the regulation of pulmonary glycogen catabolism.

Muscle PhK is a hexadecameric protein (αβγδε) with a molecular mass of 1.3 × 106 Da (for review, see Ref. 9). Calmodulin, the δ subunit, is responsible for the Ca2+-dependent activity of this enzyme; the α and β subunits regulate the activity of PhK in response to phosphorylation by cAMP-dependent protein kinase A. Glycogen phosphorylase is activated following phosphorylation by the catalytic subunit, γ, a 387-amino acid protein. cDNAs encoding muscle PhK-γ have been cloned and characterized by a number of laboratories (10–12). RNAs of 2.4 and 1.6 kilobases encoding muscle PhK-γ were

1 The abbreviations used are: PhK, phosphorylase kinase; PCR, polymerase chain reaction; NTA, nitrilotriacetic acid; bp, base pair(s); nt, nucleotide; PAGE, polyacrylamide gel electrophoresis.
detected in mouse skeletal muscle, brain, and cardiac tissues but not in liver, consistent with tissue-specific expression of the muscle enzyme (10). The tissue-specific expression of PhK may be related to the presence of distinct PhK isozymes. Consistent with this hypothesis, human and rat testis PhK\( \gamma \) (PhK\( -\gamma \)T) cDNAs have been isolated and shown to be 67% identical to rabbit skeletal muscle PhK\( \gamma \) at the nucleic acid level (13, 14). The PhK\( -\gamma \)T CDNA detected an abundant 2.0-kilobase RNA in testis which was expressed at much lower levels in most other tissues including lung. PhK\( -\gamma \)T RNA was not detected prior to 2 months of age, indicating both temporal as well as a tissue-specific regulation of expression. The purpose of this study was to identify the PhK\( \gamma \) isozyme expressed in the fetal Type II epithelial cell and characterize its tempo-spatial pattern of expression during the perinatal period.

**MATERIALS AND METHODS**

**Animals and Breeding—**Rats used in these studies were from the N2R/Mh strain derived from a random bred Wistar line found to be homozygous for liver phosphorylase b kinase deficiency (gsg/gsd). Normal time-dated pregnant Wistar controls were obtained from Charles River Laboratories. Wistar control and gsd/gsd animals were bred at the same time so each strain could be compared on the same experimental day. There was no difference in the length of gestation between control Wistars or the gsd/gsd strain; therefore, specific metabolic differences could be distinguished from the effects of gestational age.

**Isolation of Cells—**Lungs from 2-3 litters of a single gestational age (days 19–21) were removed and placed in sterile 1× Hank’s balanced salts (without calcium or magnesium), minced into 1-mm cubes, and disrupted in a solution containing 2 mg/ml collagenase (Sigma, Type IV) and 0.15 mg/ml bovine pancreas DNase I (Calbiochem). Cells were then filtered through 160-μm nylon mesh, centrifuged, and resuspended in Joklik’s modified Eagle’s medium containing 10% fetal bovine serum. Mixed cells were plated on 100-mm tissue culture plates and incubated at 37°C to promote differential adherence of fibroblasts over a 2-h period. Mixed cell cultures were plated on 100-mm tissue culture plates and incubated as above. One unit of activity catalyzes the formation of 1 μmol of phosphate per minute, assuming a molecular weight for phosphorylase b of 97,400 and that the activity of phosphorylase b in the absence of AMP = 70% of the activity in the presence of AMP (25). Activities are expressed per wet tissue or cell weight.

**RESULTS**

**Identification of PhK\( \gamma \) in Developing Lung—**The cDNA sequences encoding rat muscle (16) and rat testis (14) PhK\( \gamma \) subunits were aligned and degenerate PCR primer pairs selected on the basis of conserved regions: forward primer L, 5′-CTCTCTGTTTGCAC-\( \alpha \)TA(T)GCAC(TA)GAG-3′; nucleotides (nt) 685–705 (all nucleotide numbering in this study conforms to the reported sequence for PhK\( -\gamma \)T from mouse liver; accession number M73808); forward primer N, 5′-AACATTGT(ACG)CATCG(GA)GACCTG-3′, nt 784–804, and reverse primer S, 5′-TCCTCCTCCAC(TG)GGTGA-3′, nt 1119–1102. The L5 and NS primer pairs were used to optimize conditions for amplification of PhK\( \gamma \) using the muscle PhK\( \gamma \) cDNA (the kind gift of J. S. Chambers, Baylor College of Medicine) or the testis PhK\( \gamma \) cDNA (the kind gift of S. Hanks, Vanderbilt University Medical School) as templates for PCR (Fig. 1). Optimal PCR conditions and degenerate primer pairs were subsequently used to amplify PhK\( \gamma \) in CDNA pools that were reverse transcribed from day 19 fetal rat lung poly(A\(^+\)) RNA prepared with the FastTrack RNA isolation kit (Invitrogen, San Diego, CA). PCR products were size-fractionated by agarose gel electrophoresis, blotted onto nylon membranes, and hybridized with 10 μg of total RNA, isolated by the method of Chomczynski (38). Following an overnight incubation at 42°C, unhybridized RNA was digested with ribonuclease and the protected fragments analyzed by SDS-PAGE/autoradiography. PhK\( \gamma \)T RNA levels were quantitated by PhosphorImager analysis (Molecular Dynamics, Sunnyvale, CA). Since actin levels varied during development, a probe for rat ribosomal protein L32 (GenBank accession number X06483) was used as an internal control for gel loading and transfer.

**Spatial expression of PhK\( \gamma \)T RNA was assayed by in situ hybridization.** Sense and antisense probes were generated as described above, using \( ^{35} \)S-labeled UTP, and hybridized with cryosections of lungs with which we have previously described (19).

**Analysis of PhK\( \gamma \)T Probe Labeling—**The entire coding sequence of rat PhK\( \gamma \)T (nt 343–1563) was amplified by PCR, cloned into the bacterial expression vector pET21 (Novagen, Madison, WI), and transfected into Escherichia coli BL21(DE3) cells. Following induction with 0.1 mM isopropyl-\( \beta \)-D-thiogalactoside for 4 h, recombinant PhK\( \gamma \) was recovered by extraction of the bacterial pellet with 6× guanidine in 20 mM Tris (pH 7.9), 500 mM NaCl followed by chromatography on NTA-agarose, as described by the supplier (Qiagen, Chatsworth, CA), and purified as described previously (20). Affinity-purified antisera was prepared by chromatography over recombinant PhK\( \gamma \)T immobilized on Aminolink resin (Pierce Chemical Co.). The column was eluted with 5 mM glycine (pH 2.3), 150 mM NaCl, 0.1% bovine serum albumin and the affinity purified antisera immediately neutralized with 0.1 volume of 5× NaOH.

For Western blot analyses, lungs or Type II epithelial cell pellets were homogenized in 10 mM Tris (pH 7.5), 0.25% sucrose, 1 mM EDTA, 50 mM NaCl, and 0.1% phenylmethylsulfonylfluoride, centrifuged, and the tissue-specific expression of the tissue and isolated Type II epithelial cell was assessed by ribonucleic acid isolation (13, 14).

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Fig. 1. Isolation, cloning, and sequence of PhK-γ in fetal rat lung. A, degenerate primer pairs LS or NS were used in polymerase chain reactions to amplify sequences of 434 and 335 bp, respectively, from cDNA templates encoding rat muscle (PKM) and rat testis (PKT) phosphorylase kinase catalytic subunits. Primer pair LS was subsequently used to amplify PhK-γ in cDNA pools which were synthesized by reverse transcription of three separate preparations of poly(A)+ RNA isolated from day 19 fetal rat lung. PhK-γ fragments of 509 and 434 bp were identified, cloned, and sequenced. B, nucleotide sequence analyses of cDNA clones encoding the 434-bp fragment (open box) indicated that this fragment corresponded exactly to nt 685-1119 of the open reading frame of PhK-γ (solid box). cDNA clones encoding the 509-bp fragment were identical to the 434-bp fragment with the exception of a 75-bp insert. PhK-γ-antisense RNA expression was confirmed by: 1) hybridization with PhK-γ antisense RNA probes (see Fig. 2) or the rat muscle PhK-γ (PKM) cDNA. Labeled probes were hybridized with 10 μg of total RNA isolated from adult rat testis (T), muscle (M), lung (AL), or day 19 fetal lung (FL). Following an overnight incubation at 42 °C, unhybridized RNA was digested with ribonuclease and the protected fragments, recovered by ethanol precipitation, were analyzed by SDS-PAGE. PhK-γ RNA bands were quantitated by phosphorimager analysis of the dried gel and normalized to an internal control (L32 RNA). Results presented represent the mean ± the S.D. of four separate experiments.

Fig. 2. Expression of PhK-γ RNA in lung tissue. 32P-Labeled antisense RNA probes (pkt and pkm at right of figure) were generated from the 3′-untranslated region of the rat testis PhK-γ (PKT) cDNA (see Fig. 1B) or the rat muscle PhK-γ (PKM) cDNA. Labeled probes were hybridized with 10 μg of total RNA isolated from adult rat testis (T), muscle (M), lung (AL), or day 19 fetal lung (FL). Following an overnight incubation at 42 °C, unhybridized RNA was digested with ribonuclease and the protected fragments were analyzed by SDS-PAGE/autoradiography. A 268-base fragment is protected by PKT in all four tissues, although expression in lung and muscle is very low relative to testis; in contrast, PKM protects RNA only in muscle tissue.

Significant homology to any sequence in the GenBank database was detected in Type II cell RNA isolated from both fetal and adult rat lung (see Fig. 8B). Amplification of rat genomic DNA, with primers flanking the insert, followed by sequence analysis confirmed that this sequence represented an unspliced intron (Fig. 1C). No other forms of PhK-γ were detected with these or a distinct set of degenerate primers (NS primer pair, not shown) suggesting that the testis isozyme was the major or only form of the catalytic subunit expressed in fetal lung tissue.

Expression of PhK-γ-T RNA in Developing Lung—PhK-γ RNA levels were assessed in developing lung tissues by RNase protection using radiolabeled probes generated from the unique 3′-untranslated regions of rat testis or muscle PhK-γ. Consistent with previous reports (13, 14) testis PhK-γ was readily detected in adult testis and was expressed at much lower levels in adult skeletal muscle, adult lung, and fetal lung; in contrast, muscle PhK-γ expression was detected in skeletal muscle but not lung or testis (Fig. 2). The level of testis PhK-γ RNA in lung tissue remained relatively constant from day 17 of gestation through the postnatal period and in the adult (Fig. 3) and was similar in isolated fetal (day 20 of gestation) and adult Type II epithelial cells. In situ hybridization with antisense probes indicated that PhK-γ-T RNA was expressed predominantly in the epithelium of developing airways on day 17 of gestation (Fig. 4A); however, by day 19 of gestation virtually all cells in the lung expressed PhK-γ-T RNA and this expression profile was maintained postnataally (Fig. 4B). Overall the results of these studies suggest that the temporal-spatial expression of PhK-γ-T RNA did not vary significantly during the perinatal period.

Hybridization with control sense probes resulted in significant signal in the bronchiolar epithelium and blood vessel walls of fetal (not shown) and postnatal lung (Fig. 4E). The specificity of PhK-γ-T antisense RNA expression was confirmed by: 1) in situ hybridization with two separate sense probes from the 3′-untranslated or 3′-coding region of PhK-γ-T; 2) by in situ hybridization with irrelevant sense probes (cdk-4 and cdk-1); and 3) by RNase protection assays with labeled PhK-γ-T sense probes (Fig. 5). Antisense PhK-γ-T RNA was consistently detected in both fetal and adult lung but not in adult testis or skeletal muscle (Fig. 5). These results suggest that PhK-γ-T antisense RNA is expressed in lung in a spatially restricted manner.

Expression of PhK-γ-T Protein in Developing Lung—The en-
significant signal was observed in the epithelium of some airways in relatively little signal in day 17 gestation lung (generated from the 3'-untranslated region of the rat PK γ cDNA. In situ hybridization with antisense probes indicated that PK γ RNA was expressed predominantly in the epithelium of developing airways on day 17 of gestation (panel A). By day 19 of gestation, virtually all cells in the developing lung expressed PK γ RNA (panel B). Widespread expression was maintained in lung tissues from day 21 of gestation (panel C) and postnatal day 21 rats (panel D). In situ hybridization with sense probes resulted in relatively little signal in day 17 gestation lung (panel E); however, significant signal was observed in the epithelium of some airways in day 21 gestation lung (panel F). Sense signal was not detected with irrelevant sense probes and was also detected with a second sense probe generated from the 3' end of the coding sequence of the rat PK γ cDNA (not shown).

Fig. 5. Expression of antisense PhK-γT RNA in fetal and adult lung. 32P-Labeled sense and antisense probes were generated from the 3'-untranslated region of the rat PK γ cDNA and hybridized with 10 μg of total RNA isolated from fetal (day 17 and 21 of gestation) rat lung, adult rat lung (L), adult rat testis (T), and adult rat muscle (M). Following overnight incubation at 42°C, unhybridized RNA was digested with RNase and the protected fragments were analyzed by SDS-PAGE/autoradiography.

The glycogen storage disease (gsd/gsd) rat is incapable of mobilizing liver glycogen due to deficiency of phosphorylase kinase activity (for review, see Ref. 7) and Rannels et al. (8) have demonstrated that no net glycogenolysis occurred in the lungs of gsd/gsd fetuses. In order to determine if the testis isoform of PhK-γ plays an important role in regulation of pulmonary glycogen mobilization, expression of PhK-γT RNA and protein was characterized in the lungs and isolated Type II epithelial cells of gsd/gsd and wild type fetuses. Quantitation of PhK-γT RNA by RNPase protection assays indicated no difference in PhK-γT RNA levels in Type II epithelial cells isolated from gsd/gsd and control fetuses (Fig. 8A); furthermore, the alternatively spliced form of PhK-γT was also detected in both fetal and adult gsd/gsd Type II cells (Fig. 8B). In contrast to these results, PhK-γT protein was virtually undetectable in Western blots of gsd/gsd lungs or isolated fetal and adult Type II epithelial cells (Fig. 8C). This finding was confirmed by immunocytochemistry which demonstrated readily detectable PhK-γT protein in Type II epithelial cells of control but not gsd/gsd fetuses (not shown).

PhK Enzyme Activity in Developing Lung—PhK enzyme activity in freshly isolated fetal Type II epithelial cells (day 20 of gestation) was reduced 8-fold in gsd/gsd Type II cells (Table I). The decline in gsd/gsd PhK activity was accompanied by a 12-fold reduction in phosphorylase a activity but only a 2-fold decrease in total phosphorylase activity. These results are consistent with a major role for PhK in activation of phosphorylase in the fetal Type II cell. The corresponding decreases in PhK...
enzyme activity, PhK\-\gamma is immuno-reactive protein and glycogen degradation in gsd/gsd Type II cells are strongly implicated the testis isoform of PhK\-\gamma in the regulation of glycogen degradation during fetal lung development. PhK\-\gamma enzyme activity in isolated adult Type II cells was increased approximately 3-fold over activity in comparable fetal cells (Table I). Despite the fact that immuno-reactive PhK\-\gamma protein was virtually undetectable in isolated gsd/gsd adult Type II cells PhK\-\gamma activity was reduced only 1.4-fold relative to wild type cells. These results, coupled with the presence of significant phosphorylase a activity in adult gsd/gsd Type II cells (approximately 7-fold higher than in comparable fetal cells), suggest that a novel form of PhK\-\gamma may be expressed in the adult Type II cell.

**DISCUSSION**

The present study focused to the identification and characterization of PhK\-\gamma expression in lung and, in particular, the Type II epithelial cell during the perinatal period. PCR conditions were designed to identify both known and closely related novel isoforms of PhK\-\gamma. The collective results of PCR, RNase protection, and in situ hybridization studies indicate that the testis isoform was the only detectable form of PhK\-\gamma in fetal lung tissue. Furthermore, PhK\-\gamma RNA was readily detected in fetal Type II epithelial cells and was present at comparable levels in wild type and gsd/gsd fetuses; however, in contrast to wild type animals, PhK\-\gamma protein and enzyme activity were significantly decreased in gsd/gsd fetal Type II cells. Given these results, it is likely that the lack of PhK\-\gamma protein is directly responsible for impaired glycogen mobilization in gsd/gsd Type II cells during the perinatal period.

It is unclear if the small amount of enzyme activity detected in fetal gsd/gsd Type II cells is due to residual testis PhK\-\gamma isozyme, to contamination of the Type II cell preparation with some other cell types, or to the presence of a minor, novel PhK\-\gamma isozyme. Support for a novel PhK\-\gamma isozyme comes from the unexpected finding that PhK\-\gamma activity is significantly increased in adult Type II cells and is only slightly decreased in adult gsd/gsd Type II cells. PhK\-\gamma in adult Type II cells is immunologically distinct and is sufficiently divergent in nucleic acid sequence from the muscle and testis isoforms such that it is not detected in fetal Type II cells by PCR with degenerate primers. The identity and function of this putative novel PhK\-\gamma isozyme remains unknown but is particularly intriguing given the paucity of glycogen in adult Type II epithelial cells.

Apart from modulation of \alpha\ and \beta regulatory subunit expression, PhK\-\gamma enzyme activity in developing lung may be regulated at several levels. Differential splicing of the PhK\-\gamma gene gives rise to a least one alternatively spliced RNA transcript which was detected in fetal and adult Type II cells from both wild type and gsd/gsd animals. The alternatively spliced PhK\-\gamma transcript encodes a 75-bp in-frame insertion which corresponds positionally to the E intron of the rat muscle PhK\-\gamma

**Table I**

|                | Phosphorylase a | Total phosphorylase | Phosphorylase kinase |
|----------------|-----------------|--------------------|---------------------|
|                | units/g         | milliunits/g       |                     |
| Control fetal lung | 4.60 ± 16       | 6.17 ± 15          | 9.96 ± 5.2          |
| gsd/gsd fetal lung| 0.43 ± 0.20     | 3.99 ± 0.26        | 0.60 ± 0.31         |
| Control fetal Type II T2P | 3.76 ± 0.47     | 6.33 ± 0.89        | 12.39 ± 1.86        |
| gsd/gsd fetal Type II T2P | 0.31 ± 0.06*    | 2.70 ± 0.09*       | 1.55 ± 0.29*        |
| Control adult lung | 5.32 ± 0.13     | 6.78 ± 0.29        | 38.77 ± 3.26        |
| gsd/gsd adult lung| 1.50 ± 0.06*    | 3.51 ± 0.13        | 34.33 ± 2.62        |
| Control adult Type II T2P | 7.14 ± 0.70     | 8.85 ± 0.77        | 25.34 ± 2.67        |
| gsd/gsd adult Type II T2P | 2.36 ± 0.28     | 11.17 ± 1.42       | 20.59 ± 2.41        |

* p < .05 versus corresponding control.
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gene (27). Insertion of 25 amino acids into the catalytic domain of PhK-γT could conceivably reduce enzyme activity or alter substrate specificity. Differential splicing resulting in deletion, replacement, or insertion of specific sequences has previously been reported for muscle α and β and liver α subunits (28, 29) and may play a role in regulating PhK enzyme activity.

Spatial distribution of PhK-γT may be regulated in part by antisense RNA expression. Antisense transcripts were detected by RNase protection and were localized by in situ hybridization to portions of the bronchiolar epithelium and the blood vessel wall. There was no detectable change in antisense expression in fetal and adult lung; antisense expression was not detected in testis or muscle, consistent with a tissue-specific response. However, it was not clear from these studies if PhK-γT protein and/or enzyme activity was reduced at the site of antisense expression.

The PhK-γT RNA transcript contains a number of elements consistent with translational control of expression. First there is an AUG located at position –82, upstream from the major open reading frame, which is followed by an in-frame STOP codon at position 27–29 within the coding sequence of PhK-γT. The sequence context of the upstream AUG is similar to the authentic start codon and may therefore be recognized by the ribosome resulting in inhibition of translation (for review, see Ref. 30). Second, an RNA folding algorithm (31) predicts stable secondary structures within the 5′ leader sequence with the potential to inhibit ribosomal scanning (32). It is possible that alternate sites of PhK-γT transcription initiation (14) or differential splicing of the primary transcript may lead to loss of inhibitory elements in the leader region resulting in more efficient translation. In this regard, translational control may account for similar levels of PhK-γT protein in lung and testis despite the fact that PhK-γT RNA levels are approximately 100-fold higher in testis.

The testis isoform of PhK-γ is the major source of PhK activity in the fetal Type II epithelial cells. The importance of PhK-γT in glycogen mobilization in the prenatal lung is underscored by parallel changes in PhK-γT protein, enzyme activity, and glycogen catabolism in gsd/gsd fetal Type II cells. The tempo-spatial regulation of PhK-γT expression in fetal lung is likely to be very complex and may involve differential splicing, antisense expression, and translational controls in addition to interaction with various regulatory subunits. Despite a clear role for PhK-γT in fetal lung, the function of this enzyme and the identity of other PhK-γ isozyme(s) in postnatal lung remains unknown.

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