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Phosducin-like protein: An ethanol-responsive potential modulator of guanine nucleotide-binding protein function

(signal transduction/neural cells)

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ABSTRACT **Acute and chronic exposure to ethanol produces specific changes in several signal transduction cascades. Such alterations in signaling are thought to be a crucial aspect of the central nervous system's adaptive response, which occurs with chronic exposure to ethanol. We have recently identified and isolated several genes whose expression is specifically induced by ethanol in neural cell cultures. The product of one of these genes has extensive sequence homology to phosducin, a phosphoprotein expressed in retina and pineal gland that modulates trimeric guanine nucleotide-binding protein (G protein) function by binding to G-protein $\beta\gamma$ subunits. We identified from a rat brain cDNA library an isolate encoding the phosducin-like protein (PhLP), which has 41% identity and 65% amino acid homology to phosducin. PhLP cDNA is expressed in all tissues screened by RNA blot-hybridization analysis and shows marked evolutionary conservation on Southern hybridization. We have identified four forms of PhLP cDNA varying only in their 5′ ends, probably due to alternative splicing. This 5′-end variation generates two predicted forms of PhLP protein that differ by 79 aa at the NH_2 terminus. Treatment of NG108-15 cells for 24 hr with concentrations of ethanol seen in actively drinking alcoholics (25–100 mM) causes up to a 3-fold increase in PhLP mRNA levels. Induction of PhLP by ethanol could account for at least some of the widespread alterations in signal transduction and G-protein function that are known to occur with chronic exposure to ethanol.**

Significant advances have recently been made in understanding the biochemical and molecular changes occurring during chronic exposure of the central nervous system (CNS) to ethanol (see refs. 1–3 for reviews). There is now significant evidence suggesting that signal transduction is a major site of ethanol action. Thus, changes in calcium channels or calcium flux (4–7), cyclic AMP accumulation (8–13), protein kinase C regulation (14, 15), and phosphotidylinositol metabolism (16–19) have all been seen following chronic exposure to ethanol. Suggested mechanisms for these diverse changes in signal transduction include ethanol-induced alterations in the abundance and function of guanine nucleotide-binding proteins (G proteins) coupled to effectors such as adenylyl cyclase and phospholipase C (12, 13, 16, 17, 20–22). These findings led to the proposal that changes in G-protein function could be a central element of CNS adaptation to ethanol (20, 23, 24).

G-protein function is normally regulated by the occurrence of multiple isoforms of the α, β, and γ subunits, alterations in α-subunit abundance, and by G protein-associated proteins that modulate GTPase activity or coupling to receptor/effector molecules (see refs. 25–28 for reviews). One such G protein-regulatory protein is phosducin, a phosphoprotein originally identified in retina and pineal gland and known to interact tightly with the $\beta\gamma$ subunits of transducin (29–31). Phosducin inhibits the GTPase activity of G_o, G_i, and G_s proteins ("other," inhibitory, and stimulatory G proteins) (32) as well as that of transducin (31). Furthermore, protein kinase A-dependent phosphorylation of phosducin antagonizes this GTPase inhibitory activity (31, 32). Thus, phosducin may be a modulator of diverse G proteins that is subject to feedback regulation by phosphorylation.

Our laboratory has studied the hypothesis that coordinate alterations in gene expression ultimately mediate long-term CNS responses to chronic ethanol exposure (33–35). We have used subtractive hybridization cloning (36) to isolate ethanol-responsive genes (EtRGs) whose function may contribute to alterations in signal transduction seen with chronic ethanol exposure. In this paper we describe and sequence§ an EtRG product, PhLP (phosducin-like protein), that shows extensive homology to phosducin but that is expressed in many tissues. Our results raise the possibility that there are multiple phosducin-like molecules which selectively regulate G-protein function. Ethanol induction of PhLP expression could be expected to produce functional alterations in G protein-coupled signal transduction, such as cAMP accumulation. PhLP induction may thus provide a mechanism whereby chronic exposure to ethanol produces diverse changes in CNS signaling.

MATERIALS AND METHODS

Cell Culture. NG108-15 cells were grown as described (34) in medium containing 10% Nuserum (Collaborative Research) or in a totally defined culture medium. Cells were routinely subcultured at 7- to 10-day intervals and treated with ethanol as described in the text (see the legend to Fig. 1). Control cultures were mock-treated and handled in an identical fashion to ethanol-treated cells.

Isolation of a Rat PhLP cDNA. Commercially prepared rat brain cDNA libraries contained in λ Zap or λ Zap II vectors (Stratagene) were screened by hybridization using standard techniques. Hybridization probe was prepared from the insert portion of a mouse PhLP cDNA isolated by subtractive hybridization screening of NG108-15 cells (36). Potential full-length isolates were analyzed by DNA sequencing.

Mapping by Rapid Amplification of cDNA Ends (RACE) of PhLP cDNA 5′-End Variants. PCR was used to amplify PhLP

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Abbreviations: CNS, central nervous system; EtRG, ethanol-responsive gene; PhLP, phosducin-like protein; RACE, rapid amplification of cDNA ends; G protein, guanine nucleotide-binding protein; ORF, open reading frame.

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§The sequences reported in this paper have been deposited in the GenBank data base (accession nos. L15355 for PhLP and L15354 for the alternate 5′ end of PhLP).

cDNA 5′ ends from a rat brain cDNA library. Nested primers were located in the vector (T3 and SK primers; Stratagene) and PhLP coding region (antiM, 5′-CTGTTCTGTCTC-CAACTGC; and antiN, 5′-CTTGTCCATCATACCAC). An aliquot (1 μl) of the cDNA library was amplified for 30 cycles by using the external primers (T3 and AntiN). After removal of primers, 1 μl of this material was then amplified in a second PCR containing the internal primers (SK and antiM). The products of this second PCR were directly ligated to a TA cloning vector (Invitrogen). Clones containing inserts were then analyzed by DNA sequencing.

DNA Sequence Analysis. DNA sequence analysis was done by the chain-termination method (37) using a manual procedure (Sequenase; United States Biochemical) and by automated DNA sequencing (Biomolecular Resource Laboratory, University of California, San Francisco). Structure of the PhLP cDNA 5′-end variants was confirmed by sequencing multiple RACE clones as well as by sequencing multiple PhLP cDNA isolates obtained from a rat cDNA library different from that used to produce RACE clones.

Sequence data were compared to the nonredundant DNA sequence data bank by using the BLASTN or BLASTX programs through the National Center for Biotechnology Information (NCBI) (38) and the MACVECTOR program (Kodak–IBI). Homology alignments (see Fig. 3) for PhLP and phosducin were generated by using the MACVECTOR program.

Northern and Southern Hybridizations. Total RNA from NG108-15 cells was isolated and analyzed by RNA blot (Northern) hybridization as described (34). Nitrocellulose membranes containing poly(A)$^+$ RNA from multiple mouse tissues or *Eco*RI-digested DNA from various species were obtained from Clontech. Probes for hybridization were prepared by PCR amplification of the insert portion of PhLP cDNA contained in a pBluescript vector (Stratagene). Primer oligonucleotides were directed against the 5′ and 3′ boundaries of the PhLP coding region. [^{32}P]dCTP-labeled probes were prepared from PCR products by hexamer labeling (39). Southern "zoo blots" were hybridized and washed as suggested by the manufacturer. Potential yeast PhLP homologues were identified by rehybridization of the zoo blot at reduced stringency (50°C, 0.9 M NaCl), followed by washing at 60°C (0.3 M NaCl).

***In Vitro* Transcription and Translation.** Sense-strand RNA was transcribed *in vitro* from full-length cDNA clones coding for the original PhLP sequence (PhLP$_S$) or for a variant with an extension of the open reading frame (ORF) by 79 aa at the NH_2-terminal end (PhLP$_L$) (see Figs. 2 and 5A, respectively). The PhLP$_S$ or PhLP$_L$ RNAs were used to prime *in vitro* translation reactions with a commercially prepared rabbit reticulocyte lysate (DuPont/NEN). [^{35}S]Methionine-labeled proteins were then analyzed by SDS/PAGE followed by autoradiography.

RESULTS

Isolation of PhLP. We have used subtractive hybridization cloning to isolate EtRGs from NG108-15 neuroblastoma–glioma cells (36). Further screening of these EtRGs identified a 2.2-kb clone with an ORF that encoded a protein, PhLP, having significant homology to a portion of phosducin (data not shown). Determination of the concentration–response relationship for PhLP induction by ethanol in NG108-15 cells (Fig. 1) showed that PhLP mRNA levels increase significantly with ethanol concentrations of 25–100 mM. PhLP mRNA abundance increased to 3 times control levels with 100 mM ethanol. No change in mRNA levels for β-actin (Fig. 1) was produced by ethanol in these experiments.

Since PhLP shows a prominent induction by ethanol and appears to be a homologue of phosducin, a known modulator of signal transduction, we further characterized the structure

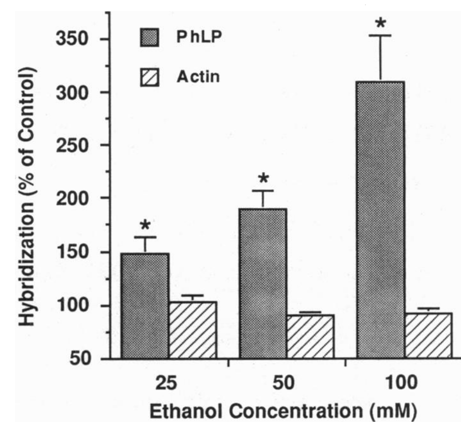

FIG. 1. Ethanol dose–response of PhLP induction. NG108-15 cells were grown with or without the indicated concentrations of ethanol for 24 hr prior to isolation of total cellular RNA. PhLP and β-actin mRNA abundance were then analyzed by quantitative Northern blot hybridization. Data are expressed as a percent of hybridization levels in mock-treated control cells. Results represent the mean ± SEM for data from three (25 and 50 mM) or seven (100 mM) independent experiments with quadruplicate determinations within each experiment. *, $P < 0.05$ by single group *t*-test analysis with Bonferroni correction for multiple groups.

and expression of PhLP. The original cDNA from NG108-15 cells was used to isolate full-length cDNA clones from a rat brain cDNA library. The DNA sequence in Fig. 2 shows a 2172-bp cDNA clone with a predicted ORF of 218 codons and a resulting protein with a molecular mass of 24.9 kDa and an isoelectric point of 4.7. The presumed initiator AUG conforms to a Kozak consensus translation start site with a purine at −3 and a guanine at position +4 relative to the "A" residue (40). The region upstream of the initiator methionine codon at base 244 contains multiple stop codons in all three reading frames. Additional cDNA isolates showed some clones to have a 3′ untranslated region ≈500 bp longer than shown in Fig. 2 because of an alternative site of polyadenylation (data not shown).

Fig. 3 shows the homology between the predicted coding region of rat brain PhLP and phosducin from several species. PhLP and rat phosducin are identical at 90 of 218 aa residues (41%) and homologous at 142 of 218 (65%) residues. The homology is across the full length of the PhLP molecule, although the COOH-terminal half of PhLP showed the strongest homology to phosducin. There are several consensus sites for protein kinase A phosphorylation in the PhLP sequence (see Fig. 2), but the well-documented protein kinase A phosphorylation site at Ser-73 of phosducin (arrow in Fig. 3) (41) is missing in PhLP.

PhLP Is Widely Expressed in Mouse Tissue and Shows Strong Evolutionary Conservation. Northern blot analysis of poly(A)$^+$ RNA from various mouse tissues is shown in Fig. 4*A*. Similar results were obtained with total RNA from rat tissues (data not shown). A major band migrating at ≈3.4 kb was seen in all tissues surveyed. Brain and kidney gave the most intense signals, whereas skeletal muscle and testis had the lowest amount of hybridization. Longer exposures of the Northern blot autoradiograms showed a faint band migrating at ≈2.9 kb in all tissues. Analysis of the 3′ end of multiple PhLP cDNA clones suggested that the major and minor bands seen on Northern hybridization were generated by alternative polyadenylylation sites (data not shown; see *Discussion* concerning Fig. 2).

Southern analysis of a "zoo blot" using a PhLP coding region probe under stringent hybridization conditions showed significant signals with all species tested except yeast (Fig. 4*B*). Lowering the hybridization stringency (0.9 M

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                                                                                          ▼
GCAAAGTGCG CTGCAGTAGA CCTCCTTGTG CCCCACGTAA GGCTATACAG AGGCCCTTGC TAACTGTGTC ATAGATACAG CAGGCTCCAT 90

GCCAATGTGA ATCCTGTCAC AGATGTCAGC GGCCCAAAGC CCCTGGTCTG TTCTCAGTTA TGCTTGTCCT ATTCAGGTCC AAAAGGGGTG 180
                                                                                                 *
ATCAATGACT GGCGCCGCTT CAAGCAGTTG GAGACAGAAC AGAGGGAGGA GCAGTGCCGG GAGATGGAGC GGCTGATCAA AAAGCTGTCT 270
                                                                     M  E   R  L  I  K   K  L  S> 9

ATGAGCTGCA GGTCCCATCT GGATGAAGAG GAGGAGCAGC AGAAACAGAA GGACCTCCAG GAGAAAATCA GTGGGAAGAT GACTCTGAAG 360
 M  S  C   R  S  H  L   D  E  E   E  E  Q   Q  K  Q  K   D  L  Q   E  K  I   S  G  K  M   T  L  K> 39

GAGTGTGGTA TGATGGACAA GAATTTGGAT GATGAAGAGT TTCTGCAGCA GTATCGGAAG CAGAGGATGG ACGAGATGCG GCAGCAGCTT 450
 E  C  G   M  M  D  K   N  L  D   D  E  E   F  L  Q  Q   Y  R  K   Q  R  M   D  E  M  R   Q  Q  L> 69

CATAAAGGGC CCCAATTCAA GCAAGTGCTT GAGATCCCCA GTGGAGAAGG ATTTTTAGAT ATGATTGATA AAGAACAGAA AAGCACCCTT 540
 H  K  G   P  Q  F  K   Q  V  L   E  I  P   S  G  E  G   F  L  D   M  I  D   K  E  Q  K   S  T  L> 99

ATCATGGTTC ATATTTATGA AGATGGTGTC CCAGGGACTG AAGCCATGAA TGGCTGCATG ATCTGCCTTG CCGCAGAGTA CCCCACTGTC 630
 I  M  V   H  I  Y  E   D  G  V   P  G  T   E  A  M  N   G  C  M   I  C  L   A  A  E  Y   P  T  V> 129
                     *
AAATTCTGCC GAGTGAGGAG CTCGGTTATT GGGGCCAGCA GTCGTTTTAC CCGGAATGCC CTTCCTGCTC TGCTCATCTA CAAGGCGGGT 720
 K  F  C   R  V  R  S   S  V  I   G  A  S   S  R  F  T   R  N  A   L  P  A   L  L  I  Y   K  A  G> 159

GAATTGATTG GCAATTTTGT TCGTGTCACT GACCAGCTGG GCGAAGATTT CTTTGCTGTA GACCTTGAAG CTTTCCTGCA GGAATTTGGA 810
 E  L  I   G  N  F  V   R  V  T   D  Q  L   G  E  D  F   F  A  V   D  L  E   A  F  L  Q   E  F  G> 189

TTGCTCCCAG AAAAGGAAGT CTTGGTGCTG ACATCTGTGC GAAACTCTGC CACCTGTCAC AGTGAAGACA GCGATCTAGA AATAGATTGA 900
 L  L  P   E  K  E  V   L  V  L   T  S  V   R  N  S  A   T  C  H   S  E  D   S  D  L  E   I  D  *> 218

ACTGATAATC CAGTTCTGTA GCTGTCTCAT TGTTTGGGCT AGAGGACACA TGTCTGTATT TATTTCTGTC CTTCCTGTCT TCTGGCTTTA 990
CAGCTGCTCT TTGTAGTCTG GTTTAGTATG TGGAAAGTCA AGAAACTCAG ATTAAATCAG AATCCTGACT CACTTTGTGG CTAGCAGTAA 1080
AGCGATTTCT AATTATATAG ACAGGAAGCT GGGTTCTTGA GCTGTTTACA TCTCTAGCGT GACATCTCTG AAATTGTTTC CAGTCAATAT 1170
TGACATGGCA CCCTTGAAGG CAATGTCTTG AAAATTGTCT TCTGATGACC TCAGAATTCC ACCAGGTCTG AGAGTAGAAT TCCCTAGTGA 1260
GTGTGTTTCT GTGCAGTGTA AACAGTGCAT TTCCATAATC ACTTGATTGC AAATCATGTT TACTTGCAAT CAGACTGTAC TTATTTTCTC 1350
CAGATCCTTT TCTACCAGGG TCCATACAAG GTTGGGAATA TAGCTCAGTG GTGAACTCTT AACCTAGCAT ACACGTAGCT CCAAGGTATC 1440
TGATTCCCAC CCAGCACTGT CAAAACTAAA GGTTCCAGAT GGTGCCTGAT GCTGTAGTTA GCAGTGGTCT CTGCTGAGGG AGTCACTGGT 1530
CCAGTCCAGC ATAATGAAGT ATGAACAGGA AGGGATGTTT TCTTGCTGAC TGCTGTCATG ATGTTGGGAA GCAGGCATCC TATTGACTGA 1620
CACTGGTGTG TATAGAGCTT GAAAACCTCA GTAGGAGGAC CTTTCTGTAT CCTGCTCATC AGCATCTTTT CCTCTGTTAT TTAGCAAGGT 1710
AATGCTTTGT TTAACCTTTT TAACTTTTTA AAAAGCTTTT TTTGCCTATT AGAAAAAAAT TCATATTTAC TAGAGGAAAA TTGGCCAAAA 1800
TACAGATGCA AAAATGGTTT AAAAATATAA TTCTGCCACC TGAATACTGT ATGTTTTGGT ACCATTCATA TATAGGGTTT TTTTTTTAAT 1890
GTATTCTAGG GTTTTTTTTT TATGGATTCA TAGGCTTTGG TTTGTCTTTA AATGACATTG GGATCATTCT GAGCATACTG TCAGATAGTG 1980
TGATCAGTTA CTAACAGAAG TTTTTCATGC TATTAAATAC TCTTCTGTAA TATTTTAATA ACTGTTGATA TTCCATTGAT TTGCTGAAAT 2070
CTGGTGTTTG GGTTTTAGAA AGATAGCAAA CTTTTTTATT ATAAACTTTC TATTATGAAA CATGTTTATC ATAGAACATA CAATAAAAAT 2160
TAAGTTAAAA AG(A)21
                                                                                       ↑
                                                                                     polyA
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FIG. 2. DNA sequence of PhLP cDNA from rat brain and the predicted amino acid sequence in single-letter amino acid code. A partial cDNA clone isolated by subtractive hybridization from NG108-15 cells was used to probe a rat brain cDNA library (Stratagene). DNA sequence analysis was done for both stands in their entirety and further verified by partial sequencing of additional cDNA isolates. Consensus protein kinase A phosphorylation sites are indicated by asterisks. The polyadenylylation signal is underlined and indicated. Approximately one-third of the PhLP cDNA isolates had an additional 500-bp stretch extending from the 3′ untranslated region (data not shown) with an additional polyadenylylation signal in the appropriate position. The site of divergence for PhLP 5′-end variants is indicated by an arrowhead (see text and Fig. 5). The star at amino acid 218 indicates the termination codon.

NaCl, 50°C) produced distinct bands with yeast DNA as well. In almost all cases, at least two bands were seen with each species when using *Eco*RI-digested DNA. Attempts to differentially melt these bands produced inconclusive results as to whether the hybridizations represented related genes or multiple fragments from a single gene (data not shown).

PhLP Possesses Multiple 5′ Ends That Generate Potential Alternate Protein Initiation Sites. The discrepancy between the size of cloned PhLP cDNAs (2.2 and 2.7 kb) and the calculated size of PhLP mRNA from Northern blot studies (Fig. 4*A*) suggested that alternate forms of PhLP might exist with differing 5′ ends. Primer extension analysis showed that although the PhLP sequence of Fig. 2 was full length, there existed at least two distinct major PhLP transcripts differing in length at their 5′ end (data not shown). Therefore, we used a modified RACE procedure to isolate clones coding for the 5′ end of PhLP. By using nested primers from the pBluescript vector and PhLP DNA sequence, PCR amplification of a rat brain cDNA library (Stratagene) produced a pattern with at least three distinct bands on agarose gel electrophoresis. Cloning and DNA sequence analysis of these PCR products showed the sequences to contain common 3′ ends that diverged at base 166 of the PhLP sequence (arrowhead in Fig. 2). Four distinct types of sequence organization were seen in multiple RACE isolates. These appeared to result from alternate splicing of at least four different 5′ exons (arbitrarily numbered segments 1–4 in Fig. 5) to the common region of PhLP (segment 5). The initial PhLP isolate depicted in Fig. 2 contains segments 1 and 5 (see Fig. 5*B*). Consensus splice-site donor/acceptor sequences exist at each of the putative exon boundaries.

The existence of these 5′-end variations was confirmed by isolation of further PhLP cDNA clones from a rat brain library separate from that used for generation of RACE clones. Sequence analysis and restriction enzyme mapping showed the same patterns as seen with the RACE clones. Furthermore, sequence analysis of these clones and RACE amplification of the 3′ region of PhLP cDNA showed no

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                                                 ↓
                         *          *          *          *          *          *          *          *
RAT PhLP       1 MERLIKKLSM SCRSHLDEEE EQQKQKDLQE KISGKMTLKE CGMMDKNLDD EEFLQQYRKQ RMDEMRQQLH KGPQFKQVLE
RAT PhD       52 _______________qmsspq srd-dKDskE rmSrKMsiqE yelihqdkeD EvcLrkYRrQ cMqdMhQkLs fGPrygfVyE>
BOVINE PhD    52 _______________qmsspq srd-dKDskE rfSrKMsvqE yelihKdkeD EncLrkYRrQ cMqdMhQkLs fGPrygfVyE>
HUMAN PhD     52 _______________qmsspq srn-gKDskE rvSrKMsiqE yelihKekeD EncLrkYRrQ cMqdMhQkLs fGPrygfVyE>
                                           ++    +    + ++    +          + +  +  ++ +  +  + + +   ++    + +

                         *          *          *          *          *          *          *          *
RAT PhLP      81 IPSGEGFLDM IDKEQKSTLI MVHIYEDGVP GTEAMNGCMI CLAAEYPTVK FCRVRSSVIG ASSRFTRNAL PALLIYKAGE
RAT PhD      117 leSGEqFLet IeKEQKvTtI vVnIYEDGVr GcdAlNssle CLAAEYPmVK FCkiRaSntG AgdRFssdvL PtLLvYKgGE>
BOVINE PhD   117 leSGEqFLet IeKEQKiTtI vVHIYEDGik GcdAlNsslI CLAAEYPmVK FCkikaSntG AgdRFssdvL PtLLvYKgGE>
HUMAN PhD    117 letGkqFLet IeKElKiTtI vVHlYEDGik GcdAlNsslt CLAAEYPiVK FCkikaSntG AgdRFsldvL PtLLIYKgGE>
                    +  ++   + ++ + + +  +  ++++  +  + +   +++++++ ++ ++    +  + +  ++    + + ++ ++ ++

                         *          *          *          *          *
RAT PhLP     161 LIGNFVRVTD QLGEDFFAVD LEAFLQEFGL LPEKEVLVLT SVRNSATCHS EDSDLEID*
RAT PhD      197 LIsNFisVae QfaEeFFAaD vEsFLnEyGL LPErEihdL>
BOVINE PhD   197 LlsNFisVTe QLaEeFFtgD vEsFLnEyGL LPEKEmhVL>
HUMAN PhD    197 LIsNFisVae QfaEeFFAgD vEsFLnEyGL LPErEVhVLe htk>
                  +  ++  +   +  + ++  +  + ++ + ++ +++ +   +
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FIG. 3. Homology between PhLP and phosducin (PhD). The predicted amino acid sequence of PhLP in single-letter code from Fig. 2 was aligned with phosducin sequences from various species by using a Dayhoff log-odds scoring matrix employed by the MACVECTOR program. PhLP sequences identical with any phosducin sequence are printed in boldface uppercase letters. Positions that were identical between PhLP and all three phosducin genes are indicated below the sequences by a plus sign. The lightface lowercase letters indicate divergence from the PhLP sequence.

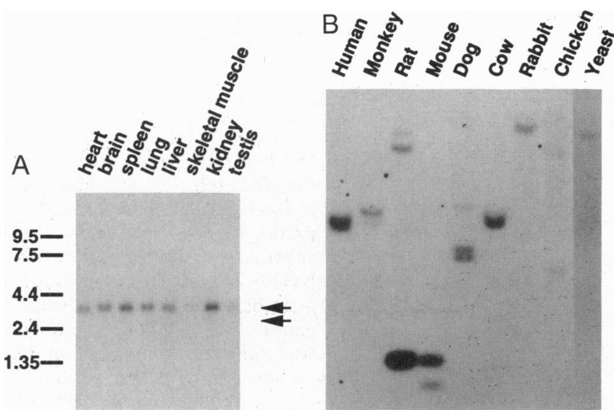

FIG. 4. Northern and Southern hybridization analyses of PhLP cDNA. (*A*) A commercially prepared (Clontech) Northern blot of poly(A)$^+$ RNA from various mouse tissues was hybridized to a probe from the coding region of rat PhLP cDNA. The position and size of molecular weight markers in kilobases are indicated on the left. Arrows denote the position of the major and minor PhLP transcripts. (*B*) A Southern blot of *Eco*RI-digested DNA from the indicated species (Clontech) was probed for PhLP cDNA sequences. All lanes were hybridized under identical conditions except for the yeast DNA lane. Hybridization conditions were as described in text.

alternate sequence patterns in this region of the molecule, other than the alternate polyadenylylation site noted above.

One 5′-end variant of PhLP cDNA, containing segments 2–3–4–5 (Fig. 5), contained an extended ORF compared to our original isolate. The corresponding protein, termed $PhLP_L$, contained a 79-aa stretch fused in-frame to the coding region of our initial sequence ($PhLP_S$). This additional peptide sequence possessed little homology to phosducin except for an 11-aa stretch (boxed region in Fig. 5*A*) that was perfectly conserved between PhLP and all phosducin sequences contained in the protein sequence data bases. The cDNA encoding this conserved sequence occurs precisely at the beginning of exon 2 in the phosducin gene (42). *In vitro* transcription followed by *in vitro* translation using rabbit reticulocyte lysates confirmed that the 1–5 (Fig. 2) and 2–3–4–5 (Fig. 5) variants of PhLP cDNA produce proteins ($PhLP_S$ and $PhLP_L$, respectively) of appropriately different sizes as predicted (Fig. 5*B*).

DISCUSSION

It is our working hypothesis that changes in gene expression could underlie the pleiotropic biochemical and pharmacological alterations that accompany adaptation of the CNS to ethanol. Here we describe the isolation of an EtRG whose product, PhLP, shows strong homology to the retinal phosphoprotein, phosducin. Based on its strong structural homology to phosducin and because phosducin is known to modulate the function of the retinal G-protein transducin, PhLP may represent a widely expressed, ethanol-responsive modulator of G-protein function.

PhLP was isolated through screening for EtRGs. The ethanol concentration–response range for induction of PhLP gene expression (Fig. 1) is similar to that seen for other EtRGs (33–35). Significant increases in PhLP mRNA abundance were seen even at 25 mM ethanol, a concentration close to the blood alcohol level (100 mg/dl, 21.7 mM) used as the legal limit of intoxication in many states. This suggests that PhLP induction is likely to occur in actively drinking alcoholics.

Detailed sequence analysis of PhLP cDNA showed the predicted protein to possess a striking amino acid sequence homology to phosducin (Figs. 2 and 3), particularly over the COOH-terminal half of the molecule. Functional domains of phosducin have not yet been determined, and no region of PhLP or phosducin has strong homology to domains of other proteins contained in sequence data bases. Thus, the regions of high sequence homology between PhLP and phosducin may aid structure–function studies on both molecules. For example, the perfectly conserved motif (Thr-Gly-Pro-Lys-Gly-Val-Ile-Asn-Asp-Trp-Arg) present in $PhLP_L$ and all known phosducin gene products (Fig. 5*A*) may be part of a functional domain. $PhLP_S$, lacking this sequence, might be expected to have an altered tissue distribution, specificity, or function compared with $PhLP_L$.

If the PhLP variants outlined in Fig. 5 are indeed products of alternate splicing, this would raise the possibility that PhLP is similar to other cases, such as developmental regu-

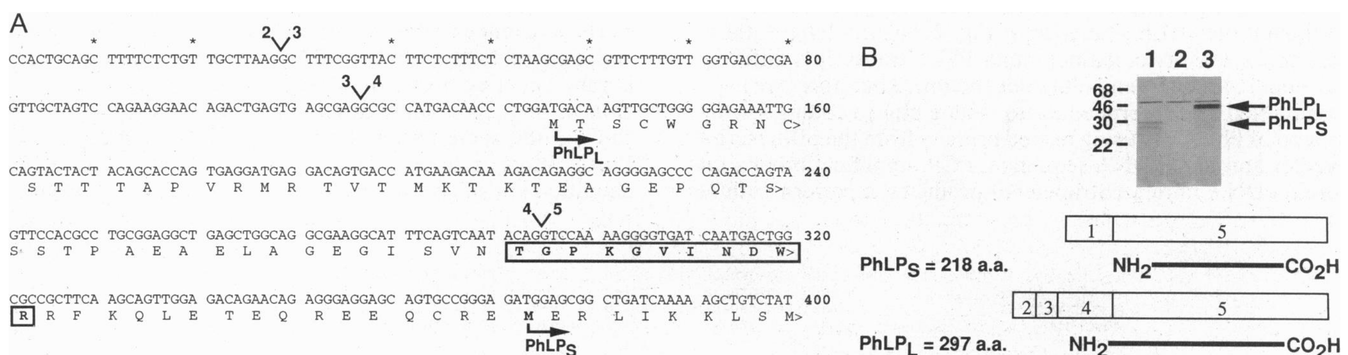

FIG. 5. Structural variation of the PhLP DNA 5′ end. (*A*) Sequence of the 5′ end of variant $PhLP_L$ DNA. RACE analysis and isolation of multiple PhLP cDNA clones from rat brain libraries showed four different sequence patterns at the 5′ end of the PhLP cDNA that appeared to result from alternative splicing. One of these was identical to the original PhLP cDNA isolate (Fig. 2; designated $PhLP_S$), while the other three contained different combinations of three presumed exons at their 5′ end. Putative exons were arbitrarily numbered 1–5 with the original PhLP cDNA isolate containing segments 1 and 5 as diagrammed in Fig. 5*B*. The DNA sequence of one of these new variants is shown (form 2–3–4–5) with an extension of the ORF of $PhLP_S$ DNA that corresponds to an additional 79 aa at the NH_2-terminal end of the product. This new predicted protein is termed $PhLP_L$. The position of presumed exon boundaries is indicated with arbitrary numbers as diagrammed in Fig. 5*B*. The predicted initiator methionines for the $PhLP_L$ and $PhLP_S$ sequences are shown by arrows. A region absolutely conserved between $PhLP_L$ and the products of all known phosducin genes is boxed. The other PhLP cDNA variants contained segments 2–3–5 and 2–5 and are not shown because their predicted coding regions are identical to that of $PhLP_S$ cDNA. (*B*) The putative exon structure of PhLP cDNA clones coding for $PhLP_L$ and $PhLP_S$ are diagrammed, and coding regions are indicated. The predicted products were confirmed by *in vitro* transcription of RNA from the appropriate cDNA clone followed by *in vitro* translation and SDS/PAGE. The photograph shows an autoradiograph of ^{35}S-labeled protein products from reticulocyte lysates containing $PhLP_S$ RNA (lane 1), $PhLP_L$ RNA (lane 3), or no added RNA (lane 2). The position of molecular weight markers $\times 10^{-3}$ is indicated on the left.

lation of the CREM (cyclic AMP-response element modulator) DNA-binding protein (43), where splicing alters the coding region and function of a protein. Preliminary Northern blot studies indeed show that PhLP transcripts containing segment 1 versus segment 4 have differing tissue distributions (unpublished data). The function of 5′-end variants of PhLP cDNA, which do not alter the coding region (Fig. 5), is unknown but may play a role in mRNA stability.

The migration of PhLP mRNA on Northern blot analyses showed a discrepancy between the calculated mRNA sizes (3400 and 2900 nt; see Fig. 4*A*) and the length of the cDNA clones (2.7 and 2.2 kb for the two polyadenylylation variants; see Fig. 2). The 5′-end variations do not resolve this discrepancy, since even the longest form (segments 2-3-4-5 in Fig. 5*A*) would only add ≈130 bp to the PhLP sequence depicted in Fig. 2. Cross-hybridization with phosducin clearly does not explain the Northern blot results, since phosducin mRNA migrates at 1300 nt (44). An extensive search for additional PhLP cDNA clones did not reveal any longer transcripts despite analysis of >15 different RACE and full-length cDNA clones. Therefore, we suspect that PhLP mRNA migrates aberrantly on denaturing agarose gel electrophoresis. This may be secondary to the long A+T-rich 3′ untranslated region, which contains several palindromic structures that could produce an altered electrophoretic mobility.

PhLP has a high degree of homology to phosducin across the entire length of both molecules (Fig. 3). Thus, it seems likely that PhLP will have a related function, perhaps with a different tissue or G protein specificity than phosducin. Phosducin has been shown to have a high-affinity interaction with $\beta\gamma$ subunits of transducin (45). The $\beta\gamma$ subunits of heterotrimeric G proteins have several different important functions. These include: binding α subunits to regenerate the G-protein complex for another cycle of activation, direct activation of effector molecules such as certain forms of adenylyl cyclase, and targeting of β-adrenergic receptor kinase to receptor molecules for desensitization (26). If PhLP acts like phosducin and modulates $\beta\gamma$ subunit function, the regulation of PhLP expression by ethanol (Fig. 1) could cause widespread changes in signal transduction.

For example, chronic ethanol exposure causes a heterologous desensitization of ligand-stimulated adenylyl cyclase activity in cultured neural cells or rat brain tissue (10, 12, 13, 21, 46) and in platelets or lymphocytes isolated from alcoholics (47–49). Bauer *et al.* (32) used *in vitro* assays to show that phosducin decreases the GTPase activity of several different G proteins and decreases adenylyl cyclase activation by β-adrenergic receptor or forskolin. These authors suggested that phosducin may interact with α subunits of G proteins in addition to its well-described affinity for the $\beta\gamma$ subunits. Thus, by analogy to phosducin, the induction of PhLP by ethanol would be expected to inhibit adenylyl cyclase activation. Several authors have suggested that changes in G-protein subunit abundance may cause the heterologous desensitization by ethanol (12, 13, 20, 21, 46). However, PhLP induction by ethanol provides an additional mechanism for ethanol regulation of cyclase function or other second messenger cascades. Thus, PhLP induction could explain many of the pleiotropic effects of ethanol on signal transduction and may account, in part, for biochemical alterations in the CNS that produce tolerance and dependence in alcoholism.

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