Absence of Pyridoxine-5′-Phosphate Oxidase (PNPO) Activity in Neoplastic Cells: Isolation, Characterization, and Expression of PNPO cDNA†

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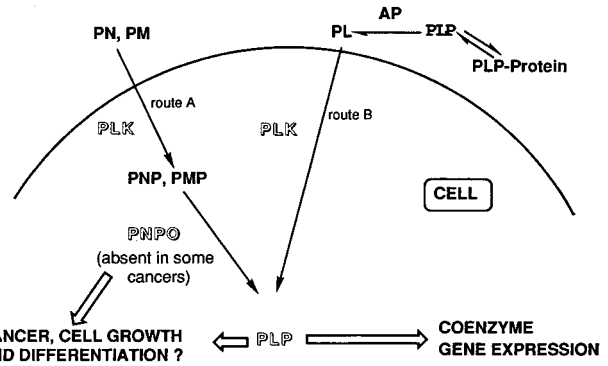
ABSTRACT: Major differences in the metabolism of vitamin B_6 in various cancers compared to their normal cellular counterparts have been documented. In particular, pyridoxine- 5′-phosphate oxidase (PNPO), the rate-limiting enzyme in pyridoxal 5′-phosphate (PLP) biosynthesis, is absent in liver and neurally-derived tumors. We show that the expression of PNPO is developmentally regulated not only in liver but also in brain. Specifically, PNPO activity in fetal brain tissue is 7.5-fold lower than that found in adult brain tissue. Furthermore, the isolation and characterization of a PNPO cDNA are described. The isolated cDNA was verified to be the authentic PNPO cDNA on the basis of two criteria. First, the translated product from the PNPO cDNA is immunologically reactive to a polyclonal PNPO antibody. Second, PNPO negative hepatoma cell lines stably transfected with the PNPO cDNA express enzymatically active PNPO protein. The availability of these biological reagents will not only facilitate in depth investigations of the reasons for the absence of PNPO in liver and brain malignancies but also aid in an understanding of the biochemical regulation of B_6 metabolism in development.

The roles of the functionally active form of vitamin B_6, pyridoxal 5′-phosphate (PLP[1]), are numerous and critical for normal cellular function. The functions of PLP include coenzymatic participation in transamination and decarboxylation of amino acids, synthesis of neurotransmitters (e.g., dopamine, serotonin, norepinephrine, and γ-aminobutyric acid), and acting as a cofactor for nearly 100 cellular apoenzymes (*1*−*3*). Other functions of PLP include the modulation of steroid−receptor interactions (*4*−*6*) and regulation of immune function (*7*, *8*). In particular, results from recent studies have implicated PLP as a negative modulator of steroid hormone receptor-mediated gene expression in vitro and in vivo (*9*−*11*, *28*).

The metabolism of PLP has been studied extensively in normal tissues by several laboratories (*12*−*18*). PLP is formed in mammalian cells by the phosphorylation of pyridoxal (PL) using PL kinase (PLK, EC 2.7.1.35) or by the oxidation of pyridoxine 5′-phosphate (PNP) and/or pyridoxamine 5′-phosphate (PMP; see Scheme 1). The synthesis of PLP from the oxidation of PNP and/or PMP requires the irreversible action of the flavoprotein pyridoxine-5′-phosphate oxidase (PNPO, EC 1.4.3.5). Several cellular mechanisms exist to tightly regulate the cellular concentration of PLP. These include the intracellular compartmentalization (i.e., "trapping") of PL by PLK-catalyzed phosphorylation, the binding of PLP to cellular proteins via Schiff base formation, the degradation of PLP to 4-pyridoxic acid catalyzed by the sequential action of nonspecific membrane phosphatases and aldehyde oxidase, respectively, and the negative feedback inhibition of PNPO by PLP (*13*, *18*).

Scheme 1: Metabolism of Vitamin B_6 and Functions of PLP[a]



[a] Dietary pyridoxine (PN) serves as the main source of pyridoxine 5′-phosphate (PNP) in liver as shown by route A. PNP is formed from PN by the action of pyridoxal kinase (PLK). PNP is then converted to the coenzymatically active form, pyridoxal 5′-phosphate (PLP), by the action of pyridoxine-5′-phosphate oxidase (PNPO). Alternatively, as shown by route B, pyridoxal (PL), derived from serum PLP by alkaline phosphatase (AP), may be directly phosphorylated to PLP. PM is pyridoxamine.

Remarkably, few studies of the status of PLP and the enzymes involved in its metabolism have been conducted in reference to neoplasia and are limited to those using a

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[1] Abbreviations: AP, alkaline phosphatase; PL, pyridoxal; PLK, pyridoxal kinase; PLP, pyridoxal 5′-phosphate; PN, pyridoxine; PM, pyridoxamine.

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Table 1: Cell Lines Used in This Study

name	description	media
C1300	mouse neuroblastoma (primary)	DMEM and 10% FCS
NB41A3	mouse neuroblastoma (C1300-derived)	DMEM and 10% FCS
NS20Y	mouse neuroblastoma (C1300-derived)	DMEM and 10% FCS
NG108	rat glioma−mouse neuroblastoma hybrid	DMEM, HAT, and 10% FCS
C6-BU1	rat glioma	DMEM and 10% FCS
SHSY5Y	human neuroblastoma	RPMI and 10% FCS
SK-N-SH	human neuroblastoma	DMEM and 10% FCS
SK-N-MC	human neuroblastoma	DMEM and 10% FCS
McA-RH7777	rat hepatoma	DMEM and 10% FCS
BRL3A	Buffalo rat liver	DMEM and 10% FCS
HepG2	human hepatoma	DMEM and 10% FCS

rodent hepatocellular carcinoma model (*19*−*22*). Interestingly, PNPO enzyme activity and protein were not detectable in several rat hepatomas (*19*−*22*). It was found that the lack of PNPO activity in these hepatic tumors followed an oncofetal developmental pattern; that is, certain liver tumors exhibited undetectable and/or low PNPO activity, comparable to or even lower than those found in fetal liver. The levels of the product of PNPO, PLP, were also significantly attenuated in these tumor-bearing animals. Furthermore, Thanassi and colleagues demonstrated a difference between the PLP-binding protein patterns present in normal and malignant liver utilizing monoclonal antibodies to the phosphopyridoxyl (PPxy) group (*23*). Recently, a novel vitamin B_6 conjugate was reported which was present in elevated levels in the plasma of cancer patients when compared with that of normal subjects (*24*).

In aggregate, these provocative results imply that major alterations in the vitamin B_6-metabolizing machinery have occurred in cancer. However, there is limited information available regarding the metabolism of this important coenzyme in other malignancies, particularly vis-à-vis PNPO activity. Given the role of PLP in neurotransmitter interconversion (*1*−*3*), it was especially of interest to examine PNPO activity in neuronally derived neoplasms. In addition, the recent findings that PLP may be involved in tissue-specific gene expression (*9*−*11*, *28*, *29*) suggest that the regulation of PNPO, the rate-limiting enzyme for PLP synthesis, deserves further investigation. In this study, we report findings demonstrating marked alterations in the PNPO status in neuronally derived tumors. Furthermore, we report here the isolation and characterization of a mammalian cDNA that encodes PNPO. Finally, the implication of results from our studies in relation to the "molecular correlation concept" put forth by Weber (*25*) will be discussed.

EXPERIMENTAL PROCEDURES

Cell Lines. The cell lines, primary rodent tissues, tumors, and culture media used in this study are described in Table 1. The C1300 primary neuroblastoma culture was generously provided by R. O'Dea, and the human hepatoma cell line, HepG2, was from S. Roy. The SHSY5Y, NS20Y, and NG108 cell lines were from P. Y. Law. All other cell lines were purchased from the American Type Culture Collection. The cell lines were maintained in a humidified atmosphere at 37 °C and 5% CO_2.

Cellular Lysates and Cytosol Preparation. The lysates used for the protein and enzyme assays were isolated from cell cultures or homogenized tissues by freezing (−150 °C) and thawing (37 °C) three times. The lysates were then centrifuged at 16000*g*, and the resulting supernatant represented the crude cellular lysate. Rat liver cytosol was prepared by centrifuging mechanically disrupted rat liver at 105000*g* for 1 h. The resulting supernatant represents the cytosolic fraction which was subsequently used for protein and enzyme activity measurements. The protein concentration was quantitated using the Bradford assay.

Pyridoxine-5′-Phosphate Oxidase (PNPO) Assay. The radioactive assay previously described by others (*26*) was employed to measure PNPO activity. The radioactive substrate *N*-phosphopyridoxal [^{3}H]tryptamine was synthesized by allowing Schiff base formation between PLP and [*ethyl*-^{3}H]tryptamine hydrochloride (NEN/DuPont, Wilmington, DE). The resulting Schiff base was covalently stabilized by reduction with sodium borohydride. The reaction mixture was applied to an AG50W-X8 column (Bio-Rad, Hercules, CA) that had been previously equilibrated with 50 mM ammonium formate (pH 4). *N*-Phosphopyridoxal [^{3}H]tryptamine ([^{3}H]PNP-tryptamine) was eluted from the column using 50 mM ammonium formate (pH 7.5), whereas the unreacted [^{3}H]tryptamine remained bound to the resin. The final specific activity of the [^{3}H]PNP-tryptamine substrate was 13 Ci/mmol.

The reaction conditions of Langham et al. (*26*) were employed to determine PNPO activity except that a smaller reaction volume (350 μL) and less protein (80 or 160 μg of total cellular protein) were employed to adapt the assay to the limited amount of protein that is available from cell cultures. Routinely, 25 000 cpm (i.e., 1.925 pmol) of *N*-phosphopyridoxal [^{3}H]tryptamine was included in the reaction. The [^{3}H]tryptamine released from the PNPO-catalyzed oxidation of *N*-phosphopyridoxal [^{3}H]tryptamine was then extracted into 2 mL of toluene. The radioactivity associated with the toluene extract represents PNPO activity. Each assay included a control which consisted of boiled rat liver protein in place of active cellular protein. The PNPO specific activity is expressed as the picomoles of substrate cleaved per hour per milligram of protein.

DT Diaphorase (DTD) Assay. The DTD activity present in the cellular extracts was measured on the basis of a previously described protocol using 2,6-dichlorophenolindophenol (DCPIP) as the substrate (*27*). Determination of DTD activity in cellular extracts was performed to rule out the possibility that the loss of PNPO activity shown in the various liver- and neurally derived tumors and tissues was due to enzymatically inactive cellular lysates. The reaction mixture consisted of 0.25 M Tris-HCl (pH 7.4), 0.2 mM NADH, 0.23 mg/mL BSA, 0.01% Tween-20, 5 μM FAD, 40 μM DCPIP, and an enzyme source. The kinetics of DCPIP reduction was measured by the negative change in absorbance at 600 nm over a 3 min period. Specific activity was then calculated using an extinction coefficient of 2.1×10^4 M^{-1} cm^{-1} and is expressed as nanomoles of DCPIP reduced per minute per milligram of protein.

Purification and Sequencing of Rat Liver Pyridoxine-5′-Phosphate Oxidase (PNPO). PNPO protein was purified from rat liver using standard biochemical and chromatographic methods (i.e., homogenization, acid fractionation,

ethanol precipitation, and DEAE-cellulose and G-150 chromatography; *20*). PNPO protein was purified to homogeneity by using a PLP-based affinity column. The affinity column was generated by allowing Schiff base formation between PLP and ω-aminooctylagarose (Sigma Chemical Co.). The PNPO protein was eluted using a PLP-containing buffer as previously described (*20*).

The purified PNPO protein was electrotransferred onto an Immobilon-P (PVDF membrane, Millipore Corp.) membrane and was visualized using a 0.1% Ponceau S staining solution. The stained band was excised and submitted for microsequencing to W. Lane of the Harvard MicroChemical Facility (Cambridge, MA). Limited tryptic digestion of the membrane-bound PNPO protein followed by separation using HPLC yielded several fragments which were evaluated for their suitability for microsequencing by mass spectrophotometric analysis. Ultimately, amino acid sequences from five tryptic PNPO protein fragments were obtained.

Isolation of Pyridoxine-5′-Phosphate Oxidase cDNA from a Rat Liver Library. Degenerate oligonucleotide primers based on the sequences obtained from the Harvard Micro-Chemical Facility were designed and synthesized in the Department of Pharmacology at the University of Minnesota (molecular core facility; funded by NIDA Grant PPG-DA08131) according to the preferred codon usage recommendations for mammalian cells as proposed by Lathe (*30*). These primers were then used in polymerase chain reaction (PCR) amplification of a rat liver cDNA library, the latter of which was constructed in our laboratory using the Pharmacia Timesaver cloning kit. Using this approach, a 261 base pair (bp) fragment of the putative PNPO cDNA was isolated. During the course of our studies, the isolation of the PNPO sequence from *Saccharomyces cerevisiae* (i.e., "PDX3"; *31*) allowed us to compare our putative partial PNPO clone with that from yeast. The 261 bp fragment was subsequently used to screen a rat liver cDNA library cloned into λ gt11 (Clontech) according to published protocols. After four rounds of screening, three potential PNPO clones were obtained. Inserts were excised from the λ arms by *Eco*RI digestion and were subcloned into the pcDNA3 vector. All three clones were sequenced twice in both directions by the dideoxy chain termination method (*32*; U.S. Biochemical Corp. sequencing kit). It was finally determined that only one clone which we have denoted as F encoded the full-length PNPO cDNA. The full-length PNPO nucleic acid sequence has been deposited in GenBank (accession number U91561).

In Vitro Transcription and in Vitro Translation and/or Immunoprecipitation. PNPO clone F and the PNPO-coding region (generated by PCR amplification) were subcloned into the pcDNA3 vector and linearized with the restriction enzyme *Xba*I. In vitro transcription reactions catalyzed by T7 RNA polymerase were then performed using the Megascript T7 kit from Ambion. As a negative control for the in vitro translation and/or immunoprecipitation experiment, PNPO clone F ligated in the incorrect orientation relative to the T7 promoter was also included. The resulting transcripts were subjected to in vitro translation reactions using a rabbit reticulocyte system employing [^{35}S]methionine labeling as recommended by the supplier (Promega, Madison, WI). The identity of the translated product was confirmed by immunoprecipitation using a polyclonal antibody to PNPO (generated in our laboratory using purified PNPO protein) and Protein A Sepharose (Pharmacia). Briefly, ^{35}S-labeled translated products in lysis buffer [25 mM Tris-HCl (pH 8.0), 0.2% NP-40, 0.02% SDS, and 0.05 M NaCl] and PNPO polyclonal antibody were mixed and kept in ice for 40 min. Protein A Sepharose slurry was then added to the above mixture and left on ice for another 1 h with occasional mixing. Samples were quick spun, and the pellet was washed sequentially with 1, 0.15, 0, and 0.05 M NaCl in lysis buffer. The final pellet was resuspended in SDS−PAGE sample loading dye and heated for 10 min at 70 °C. Samples were loaded onto a 12.5% SDS−PAGE followed by autoradiography of the gel.

Stable Transfection of the Rat Liver PNPO-Coding Region into McA. RH7777 Cells. The PNPO-coding region subcloned into the pcDNA3 vector in the correct orientation relative to the CMV promoter was linearized with *Bgl*II, a site which would not interfere with the expression of the cloned PNPO cDNA. The linearized clone was transfected into the rat hepatoma cell line McA-RH7777 cells using the calcium phosphate method (*32*). The pcDNA3 vector was also transfected into McA-RH7777 cells. Transfectants were selected using 700 μg/mL Geneticin (G418; Gibco-BRL) until such a time when no more cell death was observed. The heterogeneous population of transfectants were then cloned by the serial dilution technique (*33*).

Bacterial Expression of the PNPO cDNA Clone. The PNPO-coding region was ligated into the *Xho*I site of the bacterial expression vector pET 15b (Novagen, Inc., Madison, WI). The orientation of the resulting insert was confirmed by sequencing followed by the expression of PNPO protein by IPTG induction as recommended by the supplier. The histidine-tagged PNPO protein was purified by nickel affinity chromatography. The identity of the purified protein was confirmed by Western blot analysis using the available polyclonal PNPO antibody.

RESULTS

PNPO Activity in Neuronally Derived Tumors and Cell Lines. Previous studies reported the absence of PNPO activity in the fast-growing Morris hepatomas, 7777 and 9619A2, and in 7800, a tumor with an intermediate growth rate (*21*). Furthermore, results from these studies suggest that PNPO expression in rodent liver is developmentally regulated. We have extended these findings to include rodent neuronal normal tissues and tumors as well as neurally derived tumor cell lines. In addition to rat liver, the expression of PNPO in rat brain is also developmentally regulated (i.e., low PNPO activity in fetal rat brain and high activity in normal adult rat brain; Figure 1). The primary mouse neuroblastoma C1300 and the cell lines derived from this tumor, NS20Y and NB41A3, do not contain detectable PNPO activity within the limits of the assay. Also, the mouse neuroblastoma−rat glioma hybrid cell line, NG108-15 (*34*), and one of its parent cell lines, C6BU1 (rat glioma cells; *35*), also contain PNPO activity that is comparable to or lower than that present in fetal rat brain. Thus, analogous to rodent hepatomas, PNPO expression in rodent brain tumors follows an oncofetal developmental profile. On the other hand, the human neuroblastoma cell lines (SHSY5Y, SK-N-SH, and SK-N-MC) that were examined contain

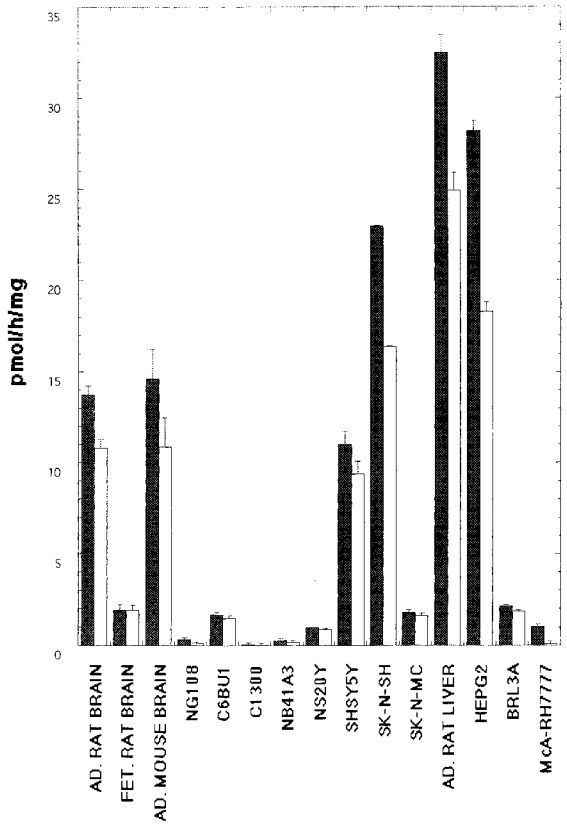

FIGURE 1: PNPO activity in various normal and neoplastic brain and liver samples. PNPO activity was assessed using the radioactive PNPO assay as described in Experimental Procedures. The black bars and white bars denote PNPO activity using 80 and 160 μg of protein, respectively. The data shown represent, from left to right, PNPO activity in neurally derived samples (11 sets) and liver-derived samples (4 sets). See Table 1 for descriptions of cell lines and tumors.

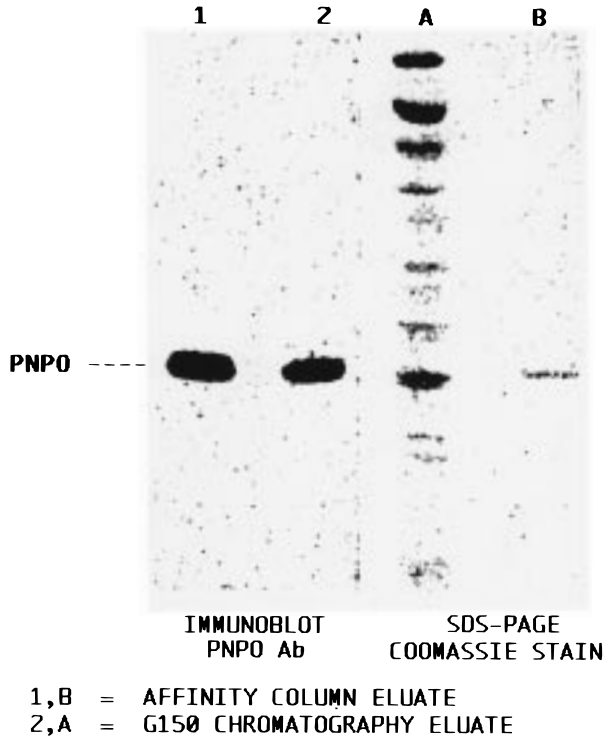

FIGURE 2: Purification of PNPO from rat liver. PNPO protein was purified as described in Experimental Procedures. Detection of the purified PNPO protein was assessed by Coomassie staining and immunoblotting with a polyclonal PNPO antibody.

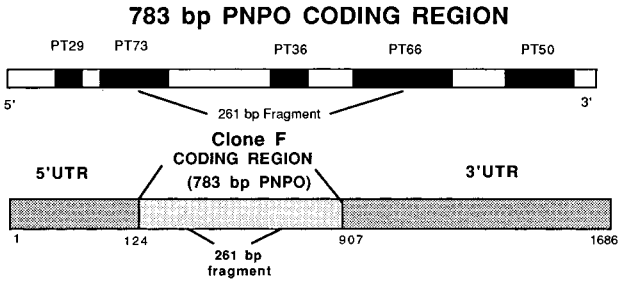

FIGURE 3: Structural configuration of the full-length PNPO cDNA (clone F).

variable PNPO activity with SK-N-MC cells exhibiting a relatively low PNPO activity. Interestingly, SK-N-SH cells have a longer doubling time than SK-N-MC cells (*36*). Whether the difference in PNPO status between these two cell lines has any bearing on their proliferative status is currently unknown.

Because the present method used to measure PNPO activity is reportedly severalfold more sensitive than the conventional colorimetric assay (*26*), we utilized this radioactive PNPO assay to reconfirm the status of PNPO in McA-RH7777 cells, a cell line established from the Morris hepatoma 7777. As expected and as shown in Figure 1, McA-RH7777 cells contain undetectable PNPO activity. Surprisingly, BRL3A cells, a cell line derived from the liver of Buffalo rat, contain relatively low PNPO activity. Interestingly, HepG2 cells, a human hepatocellular carcinoma cell line, contain relatively high PNPO activity, comparable to that found in normal human livers (data not shown). The possible explanations for these unexpected results obtained from BRL3A and HepG2 cells are discussed below. It should be pointed out that there was no correlation observed in the activities of PNPO and DT diaphorase, suggesting that the fidelity of cytosol fractions was not compromised due to destruction of enzyme activities during lysate preparation (data not shown).

Purification of PNPO from Rat Liver. We initiated the purification of PNPO from rat liver for subsequent microsequencing. Shown in Figure 2 are the purity and homogeneity of the PNPO protein that was submitted to the Harvard MicroChemical Facility. The affinity-purified PNPO protein contained one Coomassie-stainable band on SDS−PAGE (Figure 2, lane B). This protein was also immunologically reactive to the PNPO polyclonal antibody as assessed by Western blotting (Figure 2, lanes 1 and 2).

Isolation of PNPO cDNA from a Rat Liver cDNA Library. Amino acid sequences from five PNPO tryptic fragments were obtained (PTs 29, 36, 50, 66, and 73) from the Harvard MicroChemical Facility. We synthesized degenerate oligonucleotide primers based on these sequences and performed polymerase chain reactions (PCRs) using combinations of these sequences. A 261 bp putative PNPO partial cDNA was obtained using degenerate primers from PT73 (sense) and PT66 (antisense; Figure 3). We were confident that this partial cDNA clone is PNPO for two reasons. First, we were able to align four of the rat liver PNPO tryptic fragments with that of *S. cerevisiae* PNPO (PDX3). This alignment enabled us to utilize the correct combinations of primers for PCR amplification of rat liver PNPO. Second, nucleotide

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1                                                                    60
MTCGLLSVTVTFRRPAKWPGYFRHLCCRGAVMDLGPMRKSYRGDREAFEEAHLTSLDPMK

61                                                                   120
QFASWFEEAVQCPDIGEANAMCLATCTRDGKPSARMLLLKGFGKDGFRFFTNYESRKGKE

121                                                                  180
LDSNPFASLVFYWEPLNRQVRVEGPVKKLPEKEAENYFHSRPKSSQIGAVVSRQSSVIPD

181                                                                  240
REYLRKKNEELGQLYREQEVPKPEYWGGYILYPQVMEFWQGQTNRLHDRIVFRRGLATGD

241                  261
SPLGPMTHHGEEDWVYERLAP   (30.184 kD)
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___ PNPO signature pattern (I-V-E-F-W-X(5)-R-L-H-D-R)

==== potential PKC phosphorylation site [ST] - X - [RK]

FIGURE 4: Amino acid sequence of the PNPO-coding region (GenBank accession number U91561).

sequencing of the 261 bp PNPO fragment showed that PT36 is present within this fragment (Figure 3).

The 261 bp PNPO fragment was subsequently used as a probe to isolate the full-length PNPO clone by screening a λ gt11 rat liver cDNA library. Three positive clones were obtained, only one of which (clone F, Figure 3) is the full-length PNPO cDNA. Sequencing of this full-length PNPO clone revealed a 783 bp open reading frame that encodes 261 amino acids (30.164 kDa, Figure 3). The start and stop codons, ATG and TGA, which were consistent with an appropriate reading frame were identified at positions 124−126 and 907−909, respectively. There are two interesting features identified for this rat liver PNPO cDNA clone. First, the sequence motif I-V-E-F-X_5-R-L-H-D-R (Prosite PS01064), designated as the PNPO signature pattern, was found to be present in *S. cerevisiae* pdx3 (P38075), *Myxococcus xanthus* fprA (P21159), *Escherichia coli* pdxH (P28225), and *Haemophilus influenze* pdxH (P44909). We located this PNPO signature pattern in PNPO clone F close to the carboxyl-terminal region of the protein (Figure 4). Second, by using the ScanProsite program, five protein kinase C (PKC) phosphorylation sites (Prosite PS00005; [ST]-x-RK]) were identified within the PNPO clone F (Figure 4, denoted with a double underline).

Verification of the PNPO Clone F and Coding Region as a Functionally Active Protein. Verification of the isolated cDNA as a functionally active entity was absolutely necessary before any future studies could be undertaken. Thus, two criteria were established for consideration of our putative PNPO cDNA as a functionally active cDNA. (1) Transcription of the putative PNPO cDNA must produce RNA capable of supporting the synthesis of PNPO protein, and (2) the putative PNPO cDNA must be capable of expressing protein and enzyme activity when transfected into mammalian cells. The results from studies addressing these criteria are shown in Figures 5 and 6.

T7 RNA polymerase-catalyzed in vitro transcription of PNPO clone F resulted in a band of approximately 1.6 kb in a 1.5% agarose gel (data not shown). This resulting transcript was used in subsequent in vitro translation−immunoprecipitation reactions. As shown in Figure 5, in vitro translated proteins derived from both the full-length

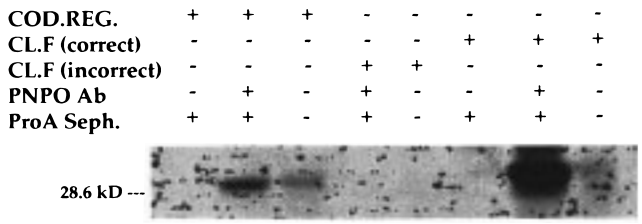

FIGURE 5: In vitro translation−immunoprecipitation reaction with the PNPO-coding region and PNPO clone F (lanes A−H reading left to right). ProA Seph. denotes protein A Sepharose beads.

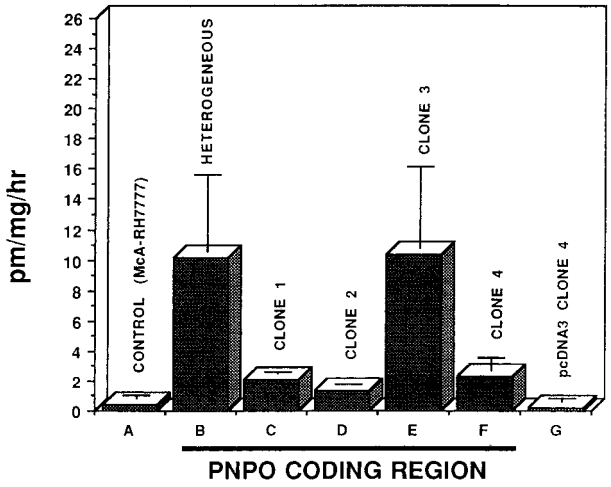

FIGURE 6: PNPO activity in McA-RH7777 and PNPO transfectants.

cDNA (clone F, correct) and the coding region of PNPO resulted in bands that comigrated with the bands that could not be immunoprecipitated by PNPO antibody (compare lanes C and H with lanes B and G, respectively). Note that the transcript derived from clone F cDNA ligated into pcDNA3 in the incorrect orientation was not translated into immunoprecipitable PNPO protein (Figure 5, lanes D and E). Results presented in Figure 5 suggest that clone F and the coding region of PNPO are both capable of generating proteins that can be recognized by the polyclonal PNPO antibody. Thus, we had satisfied our first criteria regarding the isolation of a functionally active PNPO cDNA.

To address the second issue, that the protein expressed should be enzymatically active, we stably transfected the

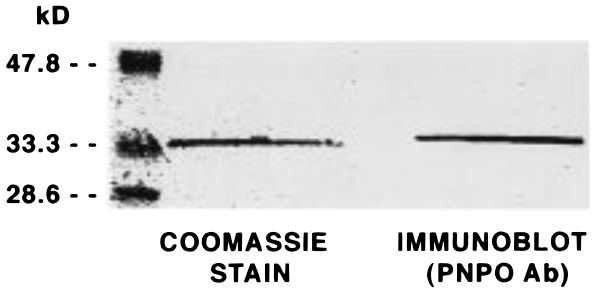

FIGURE 7: Expression of PNPO protein in a bacterially based system detected by protein staining and immunoreactivity with an antibody against PNPO.

PNPO-coding region ligated into the pcDNA3 vector and into McA-RH7777 cells. As shown in Figure 6, McA-RH7777 cells transfected with PNPO contain approximately 10.17 ± 5.04 pmol h^{-1} mg^{-1} of PNPO enzyme activity as compared to the parent cell line (0.42 ± 0.23 pmol h^{-1} mg^{-1}). Among the four transfectanted subclones, clone 3 contains a relatively high PNPO activity (10.43 ± 5.3 pmol h^{-1} mg^{-1}). McA-RH7777 cells transfected with the vector control contain PNPO activity (0.25 ± 0.13 pmol h^{-1} mg^{-1}) that is comparable to that of the parental cell line. Collectively, data presented in Figures 5 and 6 convincingly proved to us that the cDNA clone is indeed a clone that codes for PNPO.

Purification of Bacterially Expressed PNPO. In addition to using the mammalian expression system, we were able to express and purify PNPO using a prokaryotic expression system. As shown in Figure 7, the PNPO protein expressed and purified to homogeneity (lane A, Coomassie Stain) was also reactive with the polyclonal PNPO antibody (lane B, Immunoblot). It should be pointed out that the PNPO derived from the prokaryotic expression system has, as expected, a molecular mass larger than the theoretical size of PNPO. This is due to the 18 amino acids (including six histidine tags) upstream of the PNPO translation initiation site.

DISCUSSION

Unlike PLK, which is expressed in essentially all mammalian organs, PNPO is selectively present, with the highest activity found in liver, brain, and kidney (*37*). Since PLP plays a critical role in neurotransmitter biosynthesis and because of the organ-specific expression of PNPO, we recently instigated studies of the status of PNPO in neurally derived tumors and cell lines. To conduct these studies, we utilized a radioactive PNPO assay that was shown to be approximately 1000-fold more sensitive than the colorimetric assay (*26*). Implementation of this assay necessitated the synthesis and isolation of a ^{3}H-labeled *N*-phosphopyridoxal tryptamine substrate which is described in Experimental Procedures.

We have extended the original observations describing a lack of PNPO activity in liver cancer in vivo and in vitro (*19*−*22*) to include neurally derived tumor tissue and cell lines (Figure 1). As was previously shown with hepatic tissue and hepatoma, PNPO activity in rodent brain appears to follow an oncofetal developmental pattern; that is, fetal brain and several neuronally derived tumors have low and no PNPO activity, respectively, compared to normal adult brain (Figure 1). Interestingly, the BRL3A rodent cell line, was, a priori, expected to contain significant PNPO activity since it is a putatively "normal" liver-derived cell line. However, as shown in Figure 1, BRL3A contains very low PNPO activity. The ability of BRL3A to proliferate in culture suggests that these cells may have adopted a "transformed" phenotype, at least to some degree, and may explain its very low PNPO activity.

The human cell line HepG2 is a well-differentiated hepatoma which contains significant PNPO activity (Figure 1). It is possible that cellular differentiation status plays a role with respect to their PNPO expression; indeed, it was found previously that poorly differentiated rat hepatomas contained low or no PNPO activity, whereas well-differentiated hepatomas contained significant levels of this enzyme (*21*). Along this line, we have found in preliminary studies that PNPO activity can be increased in HL60 cells after treatment with phorbol ester (TPA; L. M. Nutter et al., unpublished results). Future studies of the regulation of PNPO activity vis-à-vis differentiation status are necessary and are underway. The isolation of genomic PNPO currently underway in our laboratory will greatly facilitate studies of PNPO expression.

Tumors are genetically and phenotypically heterogeneous; thus, it is possible that PNPO-positive and -negative cell types could coexist in one tumor. The culture media used to derive cell lines from such a tumor could basically "select" for a given phenotype since different media contain either PN or PL (see Scheme 1). For example, RPMI contains exclusively PN, whereas DMEM contains exclusively PL as a precursor vitamer form. Thus, it is possible that tumor cells originally possessing the PNPO-negative phenotype cannot be grown in culture media where the only precursor form is PN (i.e., RPMI). Along this line, it should be noted that proteins present in culture serum (e.g., albumin) may be capable of transporting PLP as a Schiff base for delivery to cells; subsequent dephosphorylation to PL followed by phosphorylation by PLK to PLP would bypass the need for PNPO activity (Scheme 1).

We have noted a trend with respect to PNPO activity and the media used to culture a given tumor cell line. Cells which grow in RPMI media (e.g., SHSY5Y; Table 1) may be obligated to express PNPO activity in order to synthesize the active vitamer form, PLP, since PN is exclusively the precursor form present in RPMI. In contrast, DMEM media contain PL, which allows cells to proliferate in the absence of PNPO since the cells need only to phosphorylate PL directly to PLP. Thus, it is possible that the PNPO-negative phenotype is more widespread in tumors than previously thought but that PNPO-positive tumor cells are selected by virtue of culture conditions. It should be pointed out that the bacterial δ psxH::ω null mutant (*38*) and *S. cerevisiae aux30* (*31*), both of which are PNPO mutants, failed to grow in media containing PN. We also attempted to grow McA-RH7777 cells in RPMI; however, these cells did not survive for long in RPMI (E. O. Ngo and L. M. Nutter, unpublished results). The generality of the PNPO-negative phenotype in human neoplasia absolutely requires that primary tumor tissues be studied.

Important questions are raised by the results of this and previous studies (*19*−*22*). First and foremost is addressing the mechanism operating behind the lack of and/or suppres-

sion of PNPO activity in liver- and neuronally derived tumors. Does the lack of PNPO confer growth advantage to these neoplastic cells? Is the PNPO gene mutated, and/or is its expression suppressed in malignancy? How do these tumors acquire their PLP in the face of absent PNPO activity? Thanassi and co-workers have described significant alterations in the patterns of PLP-binding proteins in liver tumors as compared to those of normal tissue (*23*); are changes in the profile of PLP-binding proteins to be found in neuronally derived tumors in addition to their counterpart normal tissues?

These provocative questions prompted us to pursue the acquisition of all the biologic reagents necessary to address these issues. Thus, as shown in Figures 3 and 4, we isolated the full-length cDNA that encodes PNPO. Rat liver PNPO exhibits a certain degree of homology to *S. cerevisiae* pdx3, *E. coli* pdxH, and *M. xanthus* fprA. In addition to confirming the identity of the cDNA clone as PNPO (Figures 5 and 7), we also demonstrated the functional integrity of this clone by stably transfecting it into McA-RH777 cells (Figure 6). We are currently characterizing these transfectants in terms of growth characteristics, including doubling times, cell cycle analysis, and growth in soft agar. The availability of these stably transfected cell lines will allow us to address the role of PNPO in the maintenance of cellular homeostasis. In particular, these cell lines will be used to investigate the role of PNPO in tumorigenicity using Buffalo rats, which are the natural host for the 7777 liver tumor. In addition, we are currently using antisense technology to develop HepG2 cell lines devoid of PNPO activity. HepG2 cells provide a good model for addressing the roles of PNPO in tumorigenicity and differentiation for several reasons. First, HepG2 cells are well-differentiated hepatoma cells; second, HepG2 is nontumorigenic in nude mice models. Thus, it will be very interesting to examine morphologic and phenotypic alterations that may result from inhibition of PNPO expression in these cells in vitro and in vivo.

Recently, studies by McCormick (*39*) suggested the facilitated tansport of PN into rat hepatocytes and kidney cells. The presence of this "putative receptor" for vitamin B_6, coupled with the five protein kinase C phosphorylation sites identified in PNPO, lead us to speculate about whether PNPO in conjunction with PLP constitutes a novel signal transduction pathway for cell growth and differentiation. The recently isolated partial PNPO genomic clone will allow us to initiate investigations of the promoter region of the PNPO gene. Accordingly, we are now poised to address the following issues. What factor(s) controls the expression of PNPO? Is the absence of PNPO expression in McA-RH7777 and NB41A3 cells due to genomic deletion? Is the lack of PNPO expression due to the absence of tissue-specific transcription factor(s) in these tumors? The absence of PNPO protein in McA-RH7777 may be due to defects at the transcriptional level. Indeed, our preliminary results indicate that McA-RH7777 and NB41A3 cells do not express PNPO mRNA, whereas, as expected, we were able to detect a PNPO transcript from our PNPO transfectants by Northern blot analysis (E. O. Ngo and L. M. Nutter, unpublished results). The availability of the PNPO gene will aid in understanding the mechanism by which PNPO expression is regulated under both physiological and pathological conditions. Furthermore, PNPO transgenic models can be developed as a scientific tool for addressing the role of PNPO in developmental biology. Along this line, expression of large quantities of the PNPO protein using the bacterial expression system and its facile purification by nickel affinity chromatography (Figure 7) is currently being taken advantage of in the production of antibodies to the PNPO protein.

The molecular correlation concept proposed by Weber (*25*) is centered around the association of the biochemical changes observed during the process of transformation and tumorigenesis. The "reprogramming of gene expression" of key enzymes in numerous cellular metabolic pathways and the accompanying changes in the concentrations of these metabolites are important determinants in neoplasia. These biochemical changes are identified by the proliferation rate, differentiation status, and the tumor grade. On the basis of these factors, we propose that the perturbations in vitamin B_6 metabolism observed in hepatomas and neuroblastomas fit within the molecular correlation concept. PNPO is the rate-limiting enzyme involved in the irreversible conversion of precursor vitamin B_6 molecules to the functionally active form PLP. The observed absence of PNPO activity in rodent hepatomas, accompanied by the alteration in PLP concentrations, correlates very well with the differentiation and proliferation status of these tumors (*21*). Importantly, we have examined the PNPO status of eight matched pair human liver samples and found that four of the eight malignant liver samples have statistically significant lower PNPO activities (E. O. Ngo and L. M. Nutter, unpublished results; tissue samples obtained from the Cooperative Human Tissue Network). Furthermore, two of the four malignant hepatomas are poorly differentiated on the basis of the histopathologic reports.

In toto, we have demonstrated that first, in addition to rodent liver, PNPO expression is also developmentally regulated in rat brain. Second, PNPO status is significantly altered in rodent neuroblastomas and glioma cell lines that were examined wherein their PNPO levels reverted back to that of the fetal state (i.e., absent). Third, the cDNA clone that we have isolated is indeed PNPO on the basis of the immunologic and enzymatic criteria we have set. With the availability of the PNPO cDNA and genomic clones, PNPO antibodies, and the transfected cell lines, we have initiated investigations of the role of PNPO in the tumorigenic process and its involvement in cellular homeostasis. In particular, the possibility of PNPO interacting with cellular protooncogenes as well as the cell cycle machinery is being explored.

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