Vol. 112, No. 1, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 15, 1983 Pages 61-65

IMMUNOBLOT DETECTION OF PYRIDOXAL PHOSPHATE BINDING PROTEINS IN LIVER AND HEPATOMA CYTOSOLIC EXTRACTS

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Received February 22, 1983

A monoclonal antibody, highly selective for the 5'-phosphopyridoxyl group, can be used to detect cytosolic pyridoxal-5'-phosphate binding proteins by an immunoblot procedure. This technique, when applied to sodium borohydrice-treated cytosolic extracts obtained from normal rat liver at various stages of development as well as several liver-derived Morris hepatomas, reveals patterns of pyridoxal-5'-phosphate binding proteins that are characteristic of the various sources of cytosol. These findings suggest that there are developmental and tumor-specific requirements for pyridoxal-5'-phosphate, the coenzymatically active form of vitamin B-6.

Although a great deal of information is available on the mechanistic aspects of vitamin B-6 catalysis (1-3) and its involvement in the intermediary metabolism of amino acids (4-6), little is known about how this essential micronutrient participates in growth, differentiation, development and tumorigenesis. It has been shown that liver-derived Morris hepatomas differ significantly from host or control livers with respect to vitamin B-6 metabolism (7). In studies with a spectrum of Morris hepatomas which ranged (8) from poorly differentiated, fast growing (e.g., #7777) to highly differentiated, slow-growing (#9618A), evidence was obtained suggesting that the metabolism of vitamin B-6 in the various Morris hepatomas fell into an oncodevelopmental

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Abbreviations used: PPxy, 5'-phosphopyridoxyl; BSA, bovine serum albumin; PLP, pyridoxal-5'-phosphate; PBS, 10 mM phosphate-buffered saline (pH 7.4); ELISA, enzyme-linked immunosorbent assay; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

0006-291X/83 $1.50

pattern in which there was "progression" (9) such that the most highly differentiated hepatoma most resembled normal rat liver (10). The patterns of vitamin B-6 metabolism found in the Morris hepatomas were shown to be tumor-specific and not characteristic of rapidly dividing hepatic tissue, such as regenerating rat liver following subtotal hepatectomy (11). With the availability of monoclonal antibodies specific for the 5'-phosphopyridoxyl (PPxy) group (12), we decided to investigate the patterns of cytosolic pyridoxal-5'-phosphate (PLP) binding proteins in Morris hepatomas in order to determine if the differences in the metabolism of vitamin B-6 found in such tumors were reflected by differences in cytosolic proteins which bind the vitamin. These studies provide the basis of this communication.

Materials and Methods

Pyridoxyl- and 5'-phosphopyridoxyl (PPxy) bovine serum albumin. Bovine serum albumin (BSA) was derivatized by incubation with excess pyridoxal or PLP followed by reduction with sodium borohydride (13-15); these materials were purchased from Sigma Chemical Co. The absorbances at 325 nm indicated that under the conditions employed, 9 and 3.7 pyridoxyl and PPxy residues, respectively, were introduced per mole of BSA.

Reduction of cytosolic extracts with sodium borohydride. Cytosolic extracts of fetal and neonatal rat liver as well as Morris hepatoma #9618A and #7794A were from a previous study and had been stored frozen (10). Regenerating rat liver from young adult male Sprague-Dawley rats was obtained fresh 24 hr after subtotal hepatectomy (11). Also obtained fresh were normal rat liver and muscle and Morris hepatoma #7777 (7). Fresh tissues were homogenized with 2 volumes of 0.25 M sucrose and the homogenates subjected to high speed centrifugation as described previously (7). To the cytosolic extracts (1.0 ml) were added dropwise with rapid stirring and ice/water cooling, 100 µl of a solution (30 mg/ml) of sodium borohydride in 10 mM NaOH. Twenty minutes after the final addition of borohydride solution, 15 µl of glacial acetic acid were added, followed by dialysis (4°C) against 10 mM phosphate-buffered saline (PBS), pH 7.4.

Selectivity of monoclonal antibody E6(2)2 for PPxy-BSA as determined by ELISA. To the wells of 96-well microtiter plates (Costar) were added 100 µl of PPxy-BSA (1 ng/µl) or pyridoxyl BSA (either 1 ng/µl or 10 ng/µl) in 50 mM carbonate "coating" buffer (pH 9.6). After 90 min at 37°C and 1 hr at 4°C, the contents of the wells were flicked out and the wells filled with a blocking solution of 1% BSA and 2.5% human plasma, previously dialyzed against buffered hydroxylamine to remove endogenous PLP (12,16), in coating buffer. After 1 hr each at 37°C and 4°C, the wells were flicked out and washed (3 times, 2 min each time) with 0.05% Tween 20 in 10 mM PBS. Following the washes, 100 µl of a solution containing monoclonal antibody E6(2)2 were added to the wells. The antibody was a 40% ammonium sulfate precipitate that had been reconstituted in 1% BSA in PBS, with the final concentrations of antibody corresponding to the original ascites fluid diluted 10^4 to 10^7-fold. After 90 min at 37°C and overnight at 4°C, the solutions containing the first antibody were flicked out of the wells which were then washed as described above. Second antibody, horseradish peroxidase conjugated goat anti-mouse IgG (Fab'_2)

from Dynatech Diagnostics, diluted 1:10,000 in 1% BSA/PBS, was added to the wells (100 µl/well). After 90 min at 37°C and 3 hr at 4°C, the contents of the wells were flicked out and the wells washed. Substrate for horseradish peroxidase was then added (100 µl/well; 15 mg o-phenylenediamine in 10 ml of 0.1 M citrate buffer, pH 5.0, containing 3 µl of 30% hydrogen peroxide). After a suitable incubation period, usually 30 minutes at room temperature, the enzymatic reaction was stopped by the addition of 50 µl of 2 M sulfuric acid. The absorbances at 490 nm were read on a Bio-Tek EIA reader.

Immunoblot detection of cytosolic PLP-binding proteins after separation by SDS-PAGE. The procedure was essentially as described previously (12).

Results and Discussion

The data provided in Fig. 1 reveal that the monoclonal antibody designated E6(2)2 is highly selective for PPxy proteins as determined by the ELISA procedure described in Materials and Methods. This highly discriminating antibody can therefore be applied to the study of hepatic cytosolic PLP-binding proteins in relation to growth, development, differentiation and tumorigenesis. To this end, we employed monoclonal antibody E6(2)2 in an immunoblot procedure (12) and examined the patterns of PLP-binding proteins in cytosolic extracts of fetal, neonatal, adult and regenerating rat liver as well as several Morris hepatoma (#7777, 7794A and 9618A). The results of the immunoblot analyses are provided in Fig. 2. It is clear from the patterns in Fig. 2

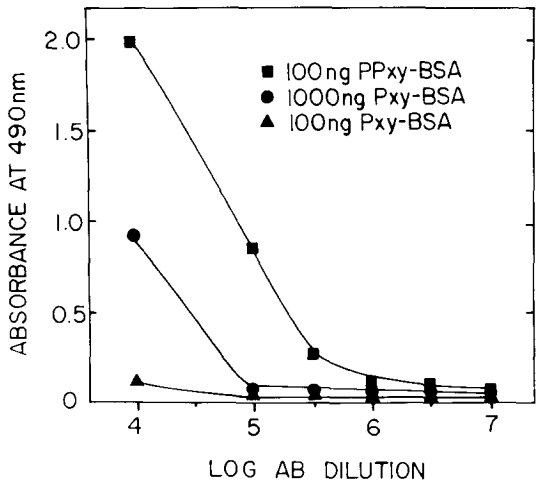

Figure 1. Selectivity of Monoclonal Antibody E6(2)2 for PPxy-BSA. ELISA determinations were carried out as described in Materials and Methods. The antigens were phosphopyridoxyl-bovine serum albumin (PPxy-BSA) and pyridoxyl-bovine serum albumin (Pxy-BSA); the amounts applied per well are provided in the figure.

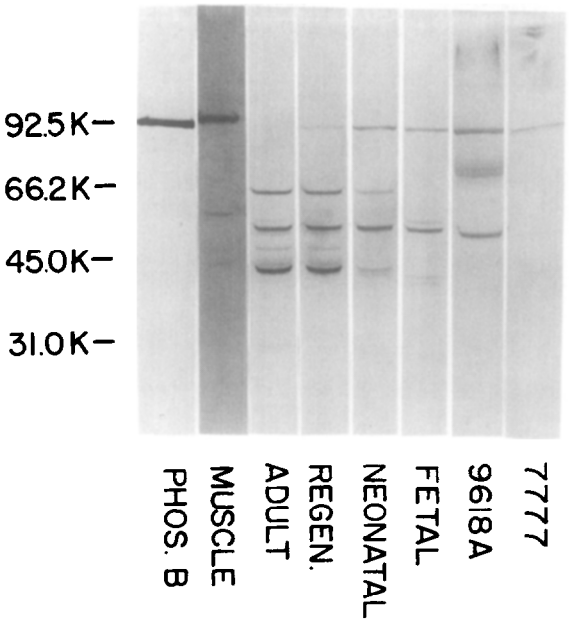

Figure 2. Pyridoxal Phosphate Binding Proteins in Rat Muscle, Rat Liver at Various Stages of Development, and Morris Hepatomas #9618A and #7777. Immunoblot analyses were performed essentially as described previously (12). Sodium borohydride-reduced phosphorylase b, containing one PPxy residue per molecule, was from a previous study (12).

that normal rat liver, in various stages of development and growth, and liver-derived tumors have distinct and characteristic profiles of cytosolic PLP-binding proteins. Accordingly, one can infer that PLP may be involved in developmental and tumor-specific roles about which there is at the present time no information. Of the normal liver samples, fetal liver has the fewest number of prominent bands which probably is a consequence of the fact that the maternal liver can provide hepatic enzymatic capabilities not yet developed in fetal liver. Of the Morris hepatomas examined, the poorly differentiated, rapidly growing #7777 has both a low PLP content (7,10) and few evident cytosolic PLP-binding proteins (Fig. 2). The most prominent band for this tumor migrates to a position coincident with a phosphorylase b marker and the expected prominent band corresponding to phosphorylase in rat muscle extracts (Fig. 2). The pattern for the highly differentiated hepatoma #9618A is particularly interesting in that it has bands which are not found to any significant degree in any of the other samples. The pattern (not shown) of

PLP-binding proteins in the well-differentiated #7794A resembles that of #7777 except for noticeable differences in band intensities, i.e., several poorly defined bands in #7777 are more sharply defined in #7794A. Significantly, none of the hepatomas resembles normal, rapidly dividing, i.e., regenerating, rat liver.

It is clear that the immunoblot procedure for the detection of PLP-binding proteins in tissue cytosols is highly discriminating and reveals significant differences among normal and neoplastic hepatic tissues. A possible interpretation for the simpler patterns seen in fetal liver and poorly differentiated Morris hepatoma #7777 is that metabolites derived from the actions of PLP-dependent enzymes may be acquired by these tissues *via* transport processes rather than made by mechanisms characteristic of and integral to terminally differentiated normal liver.

Acknowledgements

This research was supported by NIH Grants AM 25316 and AM 25490 and BRSG Grant #2-32908 from the University of Vermont College of Medicine.

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