CELLULAR LOCALIZATION OF LIGANDIN IN RAT, HAMSTER AND MAN*

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SUMMARY

Using the technique of direct immunofluorescence, ligandin, an abundant soluble organic anion binding protein was localized to the cytoplasm of hepatocytes, proximal renal tubular epithelium, and epithelial cells of the proximal, mid and distal small intestine of rat, hamster and man.

The localization and abundance of ligandin, variety of organic anions that are bound, and its activity as a gluthathione transferase suggest that this cytoplasmic protein is important in determining the net intracellular content of organic anions and in nonoxidative detoxification.

INTRODUCTION

Ligandin has been purified from rat and human liver and is a basic protein (pI 9.1) of 46,000 daltons with two apparently identical monomeric subunits (1-3). In the rat, ligandin is identical with hepatic azocarcinogen binding protein (2,4), estrogen binder I (2,4) and GSH transferase B (5). Ligandin noncovalently binds many organic anions including bilirubin, various dyes, metabolites, drugs and hormones, and covalently binds several carcinogens or their metabolites (6,7). Monospecific precipitating antibodies were prepared and utilized to quantitate rat and human ligandin in various tissues (2). By quantitative radial immunodiffusion, ligandin was detected in the supernatant fraction of homogenates of rat, human and hamster liver, kidney and small intestinal mucosa, and was absent in other tissues including serum, bile and urine (2). In the rat, ligandin accounts for approximately 5%

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of hepatic, 2% of kidney and 2% of small intestinal mucosal supernatant protein (2). Developmental, phylogenetic and pharmacologic studies support the hypothesis that ligandin is a major determinant of net organic anion flux in liver (6-10) and kidney (11).

To determine the cellular localization of ligandin, monospecific anti-rat and anti-human liver ligandin IgG were utilized for direct immunofluorescent studies.

MATERIALS AND METHODS

Ligandin was isolated from rat and human liver following homogenization in 0.25 M sucrose, 0.01 M sodium phosphate pH 7.4, centrifugation at 100,000 x g and sequential chromatography of the supernatant fraction on triethylaminoethyl cellulose, Sephadex G75 and QAE-A50 (1-3). Purity of ligandin was established by polyacrylamide gel electrophoresis with and without β mercaptoethanol, isoelectric focusing on gels and columns, and by a single zone of precipitation when reacted against a polyvalent rabbit antiserum to hepatic cell proteins. Antiserum against rat ligandin demonstrated cross-reactivity with hamster liver supernatant and yielded a single precipitin band on immunodiffusion analysis (13).

Adult male goats received multiple intradermal injections of 50-75 µg of rat or human ligandin suspended in 1.0 ml of saline and emulsified with complete Freund's adjuvant (Difco Lab., Detroit, Mich.) with 10 mg of Mycobacterium butyricum. Re-immunization at 3 weeks was with 75-150 µg of ligandin in incomplete Freund's adjuvant. Antiserum was separated from blood obtained at weekly intervals. Immunodiffusion experiments showed a single line of precipitation against ligandin and liver, kidney and small intestinal mucosal supernatants (2,3). Tetramethylrhodamine isothyocyanate (Baltimore Biol. Lab. #9091431) derivatives of immune IgG from antisera were prepared (14,15). The A515/A280 nm ratio of the conjugates ranged from 2-3, and the solution used for immunofluorescent studies contained 0.1 mg of protein per ml.

Tissues were obtained from adult Sprague-Dawley rats of either sex, fetal Sprague Dawley rats of various gestational ages, and normal adult Golden hamsters of either sex. Tissue sections were prepared from liver, kidney and proximal, mid and distal thirds of the small intestine, and rat or hamster brain, thyroid, thymus, lung, skin, myocardium, striated muscle, fat, spleen, stomach, pancreas, colon, lymph nodes, testes and ovary. Normal human liver and kidney were obtained at post-mortem examination from patients dying of diseases not involving these organs as well as from aspiration biopsy of liver. Rats bearing Morris hepatomas #77948, 777, 7794A, 8633 were also studied and primary tumors and metastatic lesions were sectioned. All tissues were frozen on dry ice and 4 micron sections were prepared using an International Cryostat, air-dried, submerged in 95% ethanol at 4°C for 5 minutes, and rehydrated in .01M phosphate buffered saline, pH 7.2 with two changes for 2.5 minutes each. Slides were immersed in 0.05% sodium nitrate - 1% acetic acid for 1 minute, transferred to 1% acetic acid for 1 minute, rewashed in buffered saline (16), stained with immunofluorescent reagents for 30 minutes at room temperature, washed five times with buffered saline for a total of two hours at 4°C, mounted with 75% glycerol in buffered saline, and viewed and photographed using a Universal Zeiss microscope employing a 580 reflector incident light system with interference filters KP600 and FI546 and barrier filter 58. Specificity of immunofluorescent staining was verified by lack of staining of non-ligandin-containing tissues, blocking of fluorescence by prior incubation of hepatic tissue sections with 3% goat anti-ligandin not derivatized with the fluoro-

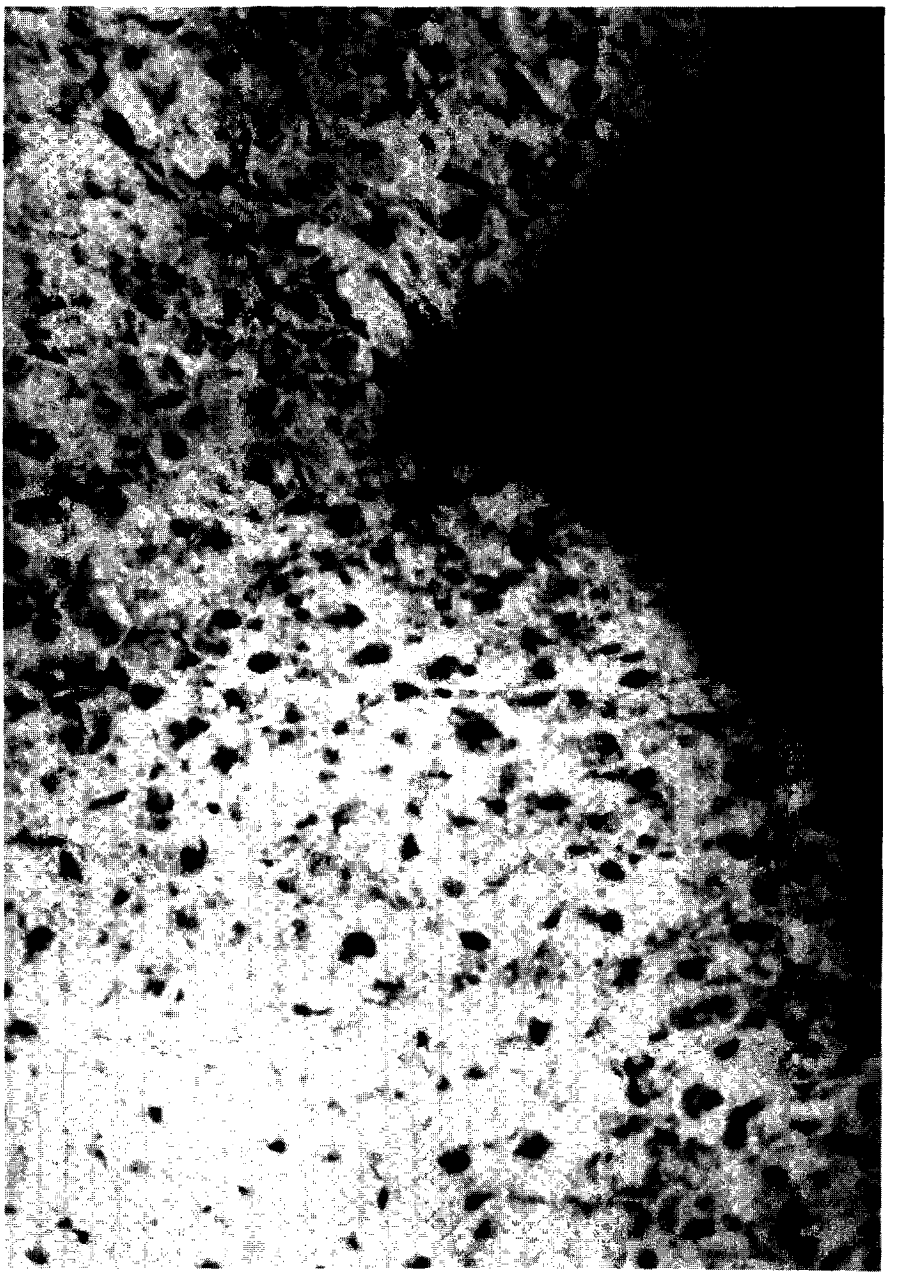

Figure 1 Localization of hepatic ligandin is evidenced by rhodamine conjugated anti-rat ligandin fluorescent staining of normal Sprague Dawley rat liver. Cytoplasmic staining is observed in hepatocytes. Fluorescence is not observed in Kupffer cells or the portal triad (upper right of the photomicrograph) (X120).

Figure 2 A representative photomicrograph of fluorescent staining of Sprague Dawley rat kidney by rhodamine conjugated anti-ligandin prepared against rat hepatic ligandin. Fluorescence is observed in tubular cells. Absence of fluorescence is noted in flomeruli and adjacent vascular structures (X240).

chrome, and sequential absorption of specific rhodamine conjugates with ligandin and removal of precipitated complexes by centrifugation.

RESULTS

All hamster, rat and human hepatocytes revealed uniform cytoplasmic staining with the anti-ligandin fluorochrome without specificity within the hepatic lobule. A representative illustration from rat liver is shown in Figure 1. No fluorescence was observed in Kupffer, stroma or bile duct epithelial cells. Fluorescence was inhibited by absorption of conjugated anti-ligandin with 2.4 molar excess of ligandin and when sections were incubated with non-conjugated immune globulin. A low level of background fluorescence was observed in all hepatic cells and was unaffected by absorption with purified ligandin. Immunoreactivity was absent in fetal rat liver during the first two-thirds of gestation. Specific immunofluorescence was faint in rat liver during the last week of gestation and immediately after birth, and increased during the first week of life to the adult level.

In the kidney, specific immunofluorescence to ligandin in rat, hamster and man was confined to cortical tubular epithelial cells (Fig. 2). No difference in immunofluorescence was observed between the P 1, 2 and 3 segments of the proximal renal tubule.

Immunofluorescent studies of the gastrointestinal tract yielded identical results in rat, hamster and man (Fig. 3). Immunofluorescence was most intense in epithelial cells of the duodenum through mid-jejunum. Epithelial cells in the distal small bowel revealed decreased specific fluorescence. Epithelial cells facing the intraluminal border exhibited intense fluorescence; no reactivity was observed in supporting tissues or goblet, argentaffin or submucosal cells. Control studies, previously described for hepatic cells, verified the specificity of fluorescence. No immunofluorescent staining of rat gastrointestinal tissues was observed in early or late rat fetuses.

The soluble fraction of extracts of Morris hepatomas (77948, 777, 7794A, 9633) yielded antigenically active ligandin. Specific immunofluorescence was

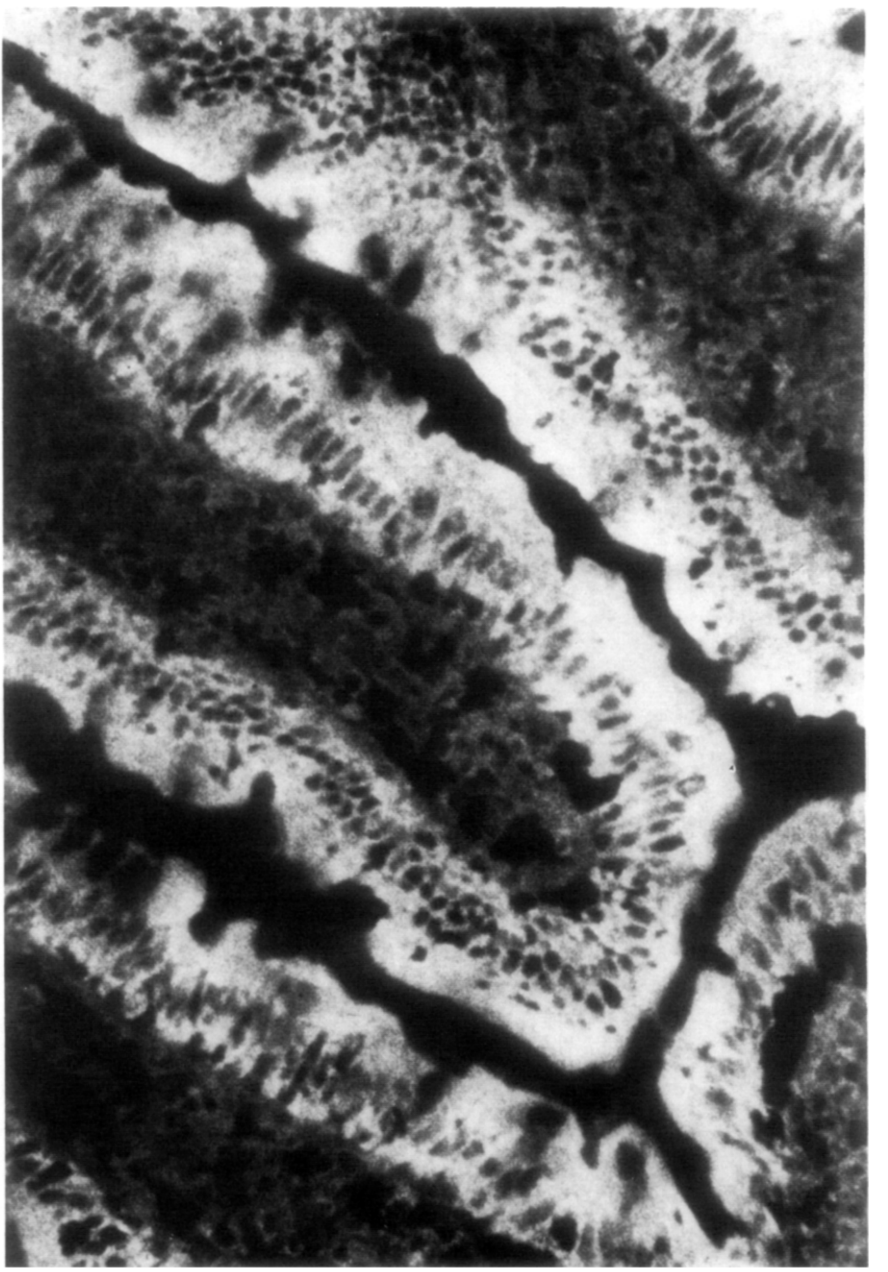

Figure 3 Fluorescence of rhodamine anti-rat ligandin in Sprague Dawley mid jejunum. Epithelial cells adjacent to the luminal border show fluorescence while other areas of the villus fail to react with the staining reagent (X120).

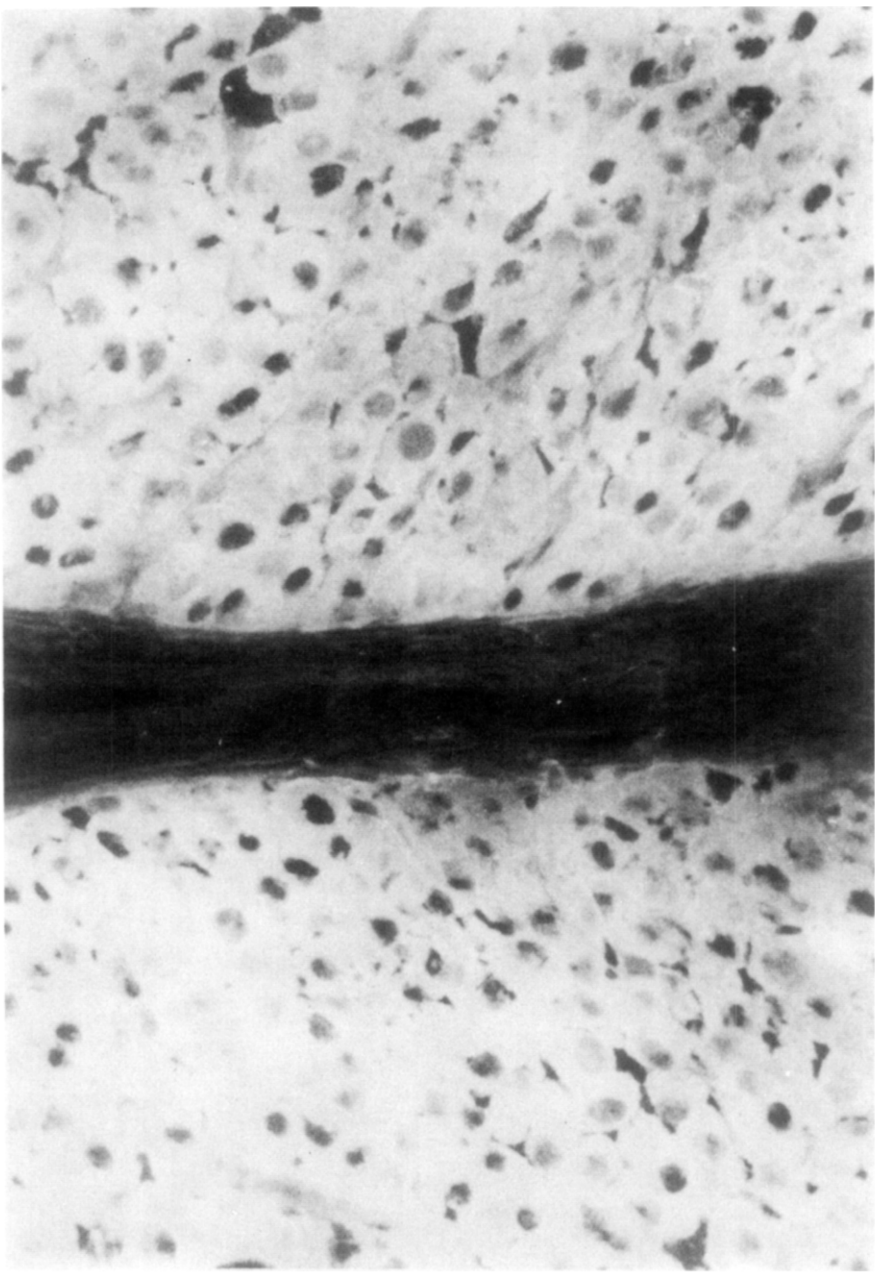

Figure 4 Fluorescence of rhodamine anti-rat ligandin of Morris hepatoma #77948 from the inoculation site in a recipient rat. A non-staining blood vessel is observed in mid-field. Staining of tumor cells is diffuse without nuclear or membrane localization (X240).

observed at sites of primary inoculation of the tumor (Fig. 3). Metastasis near the implantation area exhibited weak immunofluorescence. When tumor development progressed for at least two months, no anti-ligandin immunofluorescence was detected in metastatic sites, such as the lung.

Specific immunofluorescence was not found in any other rat or hamster tissue studied.

DISCUSSION

These immunofluorescent studies with monospecific antibodies in rat, hamster and man localize ligandin to all parenchymal liver cells, renal proximal tubular cells, and non-goblet mucosal cells of the small intestine. Other tissues did not reveal specific immunofluorescent staining for ligandin. Preliminary immunologic studies of rat liver organelle and plasma membrane fractions reveal absence of ligandin by immunofluorescence and immunoprecipitation. These observations are consistent with studies indicating that ligandin is largely, if not exclusively, cytoplasmic (2,6). Previous immunological and physiochemical studies revealed identity between Y protein (2) and the basic azo-dye binding protein of Ketterer (4) resulting in the term "ligandin" being applied to these proteins. Bannikov et al studied immunofluorescent cellular localization of antiserum to rat liver azo-dye binding protein (17). Immunofluorescent reactivity was observed in the periportal zones of rat liver and in kidney, small intestine, ovary and testes (17). While the differences between the present studies and those of Bannikov et al cannot be resolved without exchange of reagents, the specificity of our immunofluorescent conjugates was verified by several different techniques.

Immunoquantitation studies in rat, monkey and man reveal maturation of hepatic ligandin following birth (3,8). The present study of fetal rat liver confirms these observations, and indicates that development of ligandin is restricted to parenchymal liver cells, begins just prior to birth, and reaches maturity within the first 7-10 days.

Immunofluorescent staining for ligandin was uniform at the sites of

primary inoculation of the four Morris rat hepatomas studied. Metastatic lesions had reduced or absent specific staining suggesting that these cells lose the ability to synthesize ligandin. Hepatomas induced by azo-carcinogen also have reduced or negligible amounts of ligandin (18).

Ligandin constitutes 2% of supernatant protein in rat or human kidney (2,3). Because ligandin is restricted to the proximal tubular cell of the kidney, and this cell constitutes approximately 50% of kidney protein, the actual concentration of ligandin in the proximal tubular cells approximates that in liver (i.e. 5%) (2,3).

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