Separate Identities of Ligandin and the h-Protein, a Major Protein to Which Carcinogenic Hydrocarbons Are Covalently Bound.[1]

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Summary. There is a protein moiety in the C3H mouse liver cytosol which gives a line of identity with rat liver ligandin one of three azo dye binding proteins of the liver using anti-rat ligandin. This mouse liver protein has been termed mouse ligandin and is not the h-protein, the major target protein in the mouse liver of methylcholanthrene and its metabolites. Mouse ligandin is identical to a minor methylcholanthrene binding protein species that was found previously to consist of basic proteins II and III. Both mouse ligandin and mouse h-protein contain glutathione S-transferase activity with different substrate specificities.

Upon interaction with target tissues and transformable cells in culture, carcinogenic hydrocarbons become metabolically activated (1,2) and subsequently react covalently with cellular proteins, RNA and DNA (3-8). A major protein target belonging to the electrophoretic "h" family has been extensively purified from mouse skin (8,9), transformable cells in culture (10,11) and mouse liver (9). Polycyclic hydrocarbons bind covalently to this protein, which has been termed the h-protein,

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[1]Abbreviations used are: MCA, 3-methylcholanthrene; GSH, glutathione; CM, carboxymethyl.

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with a high degree of specificity and in proportion to their carcinogenic activities (4).

Similarly, it has been demonstrated that azo dye hepatocarcinogens bind covalently to three rat liver proteins that have sedimentation coefficients of 4.7S (12,13), 3.5S (13,14) and 1.7S (13,14). The 4.7S protein, also called the "slow h_2-5S azoprotein", is associated with the greater part of the bound azo dye and has been purified by Sorof et al (12), Tokuma and Terayama (15) and Ketterer et al.(16). The 3.5S protein has been purified and characterized by Ketterer et al. (13) and Morey and Litwack (17), and has been studied by Arias and his collaborators (18). It has been named ligandin because of its wide range of binding affinities towards carcinogens and their metabolites, anionic steroid metabolites, bilirubin, haem, and a number of exogenous anions (19). Rat ligandin and mouse h-protein have many similar physical properties (8,9,13,14). Both have molecular weights in the region of 40,000, a sedimentation coefficient of 3.5S, and both consist of two equal subunits of approximately 20,000 daltons. The major difference between them is their isoelectric points, rat ligandin consistently giving higher values (8.4 - 9.3) (19) than mouse h-protein (8.0 - 8.6) (9). Singer and Litwack (20) demonstrated that rat liver ligandin (known then as corticoid binder I) binds metabolites of methylcholanthrene (MCA), a potent carcinogenic hydrocarbon.

On this basis, it has been suggested that the mouse h-protein may be mouse ligandin (12). In this communication, we submit evidence that although there is a protein in the mouse liver that is immunologically identical to rat liver ligandin, it has a separate identity from the h-protein.

MATERIALS AND METHODS

Immunoelectrophoresis and Immunodiffusion Experiments: The microimmunoelectrophoresis method of Scheidegger (21) was used to identify in the mouse liver cytosol a protein identical to rat liver ligandin using antiserum raised in the

rabbit against rat liver ligandin. Double radial immunodiffusion experiments were carried using the Ouchterlony Method (22). In these experiments, rat liver ligandin was compared to the mouse liver h-protein, basic proteins II and III which bind methylcholanthrene covalently in vivo. Those proteins were purified as described previously. (9).

Separation of Mouse liver h-protein and mouse ligandin: C3H mice were injected with [^{3}H]MCA as described in a previous communication (9). The 40,000 molecular weight basic protein fraction was obtained (9), subjected to ammonium sulfate fractionation (65%), equilibrated with 10mM K phosphate buffer (pH 6.8) containing 30% glycerol, and then applied to a carboxymethyl (CM)-cellulose column (2x20 cm). When fifty 6 ml fractions had been collected by elution with the phosphate buffer, a KCl gradient was initiated. The h-protein was excluded immediately from this column, whereas ligandin was eluted by the KCl gradient.

Glutathione S-Transferase Activities: The glutathione S-transferase activities were determined using the following substrates: 1-chloro-2,4-dinitrobenzene, 3,4-dichloronitrobenzene (Aldrich) and 1,2-epoxy-3-(p-nitrophenoxy)propane (Eastman Kodak). The assays were carried out spectrophotometrically as described by Habig et al. (23).

RESULTS AND DISCUSSION

The presence of ligandin in mouse liver has already been implied from binding studies (24), and its existence is given further support by the observation that a protein in mouse liver cytosol cross-reacts with anti-rat ligandin (25). This observation has been confirmed in the present work as shown in Fig. 1, which depicts an immunoelectrophoresis (21) in which mouse liver cytosol gave a line identical to rat ligandin when the antiserum was anti-rat ligandin. However, when purified h-protein derived from either mouse liver or mouse skin was tested by Ouchterlony immunodiffusion (22) against anti-rat ligandin no precipitin reaction was

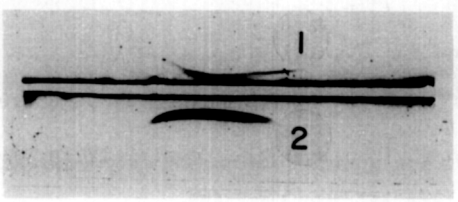

FIGURE 1

Immunoelectrophoresis of mouse liver cytosol and rat ligandin, using anti-rat ligandin in the antiserum trough. Mouse liver cytosol was placed in well 1 and rat ligandin in well 2. The micromethod of Scheidegger (21) was used. Electrophoresis was for 2 hr at 90V in 0.02M Na barbiturate buffer (pH 8.2).

obtained (Fig. 2a). This indicates that mouse h-protein is not mouse ligandin. Further evidence for the difference between mouse h-protein and rat ligandin comes from their amino acid compositions, shown in Table 1. There are several striking differences, e.g. in glycine and histidine content, which are greater than would be expected from interspecific variation in the proteins.

A more basic fraction of 40,000 daltons from mouse liver, which binds MCA to a lesser extent than the h-protein, was also tested with anti-rat ligandin and was found to give lines of identity with rat ligandin (Fig. 2b). Like rat ligandin (27), this fraction can also be resolved into two components on gradient elution from a CM-cellulose ion exchange column (9), and is apparently mouse ligandin.

The h-protein and ligandin have physical properties similar to a group of rat liver enzymes, the glutathione (GSH) S-transferases (EC 2.5.1.18) (23), which are involved in the detoxification of various foreign compounds by converting them to GSH conjugates. Rat ligandin has been shown to be identical to GSH S-transferase B (28). Mouse liver h-protein and mouse ligandin preparations both contain GSH S-transferase activities. As shown in Fig. 3, the mouse h-protein and mouse ligandin are completely

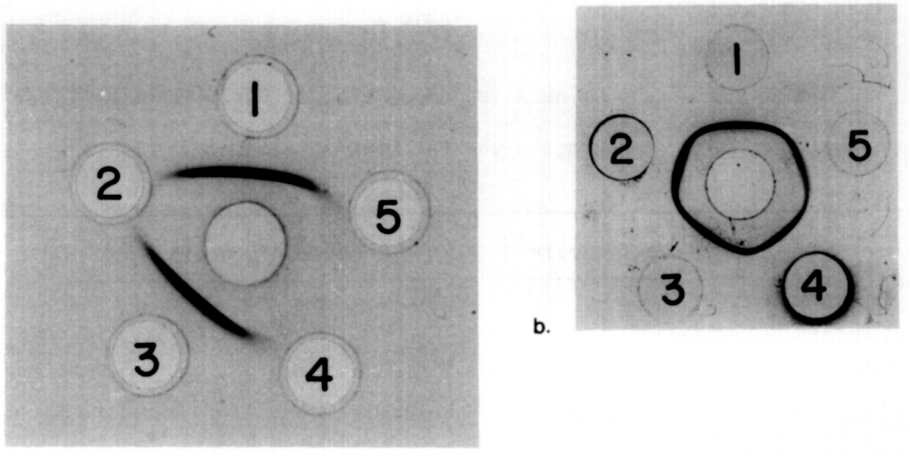

FIGURE 2

The immunological reaction of mouse liver h-protein and rat liver ligandin using the radial immunodiffusion method (22) with antiserum raised in the rabbit against rat liver ligandin. The center wells have the antiserum (5 µl each). a) Wells 1 and 3 contain 5 µl of rat liver ligandin (0.5 mg/ml); wells 2, 4, and 5 contain 10 µl of 0.5, 1 and 2 mg/ml solution of mouse liver h-protein respectively. b) Wells 1 and 3 contain rat liver ligandin, wells 2 and 5 contain 10 µl of a 1 mg/ml solution of mouse liver basic protein II, and well 4 contains 10 µl of a 1 mg/ml solution of mouse liver basic protein III. The mouse liver proteins were purified as described previously on a CM-cellulose column (9).

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separated on a CM-cellulose column. At this stage of purification both proteins contain high GSH S-transferase activities against several substrates. Although we have been able in subsequent purification steps to separate the 3,4-dichloronitrobenzene and the 1-chloro-2,4-dinitrobenzene GSH S-transferase activities from the h-protein (Sarrif and Heidelberger; Ketterer unpublished observations), we have not yet succeeded in removing all of the activity with 1,2-epoxy-3-(p-nitrophenoxy)-

Table I

Comparison of the Amino Acid Composition of Purified Mouse Liver h-Protein and Rat Liver Ligandin

	rat ligandin[19]	mouse h-protein
Cys	4	6
Asp	34	41
Met	14	9
Thr	11	15
Ser	13	13
Glu	39	40
Gly	18	38
Ala	26	28
Val	17	18
Ile	19	18
Leu	46	31
Phe	17	12
Lys	33	24
Arg	21	11
Pro	16	12
His	5	1

The amino acid analysis was determined after oxidation of the samples with performic acid as described by Moore (26).

propane as substrate. It is possible that GSH S-transferase activity is intrinsic to the h-protein, and this might be pertinent to the detoxification of carcinogen metabolites as suggested by Kuroki and Heidelberger (10). This point is being further investigated.

The finding that the h-protein is not identical to ligandin is important. Binding to ligandin is apparently found in both carcinogenic hydrocarbon

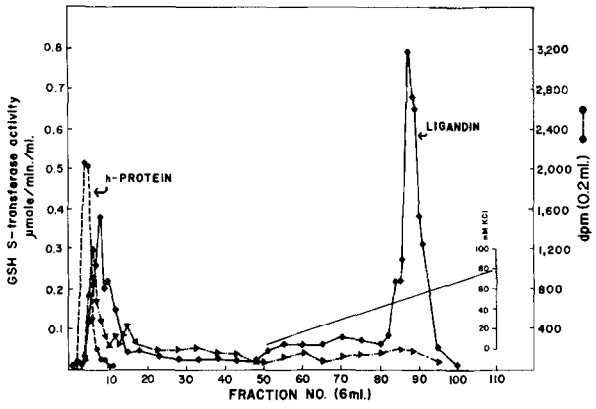

FIGURE 3

Separation of the h-protein and ligandin from mouse liver on a CM-cellulose column (2x20 cm). The mice were injected with [^{3}H]MCA as described previously (9). The basic protein fraction of the cytosol was obtained (9), subjected to ammonium sulfate fractionation (65%), and then the 40,000 dalton fraction containing the radioactive protein (25 mg), previously equilibrated with 10 mM K phosphate buffer (pH 6.8) containing 30% glycerol, was applied to the top of the column. Fifty 6 ml fractions were collected by elution with the phosphate buffer. Then a KCl gradient (75 mM) was initiated. The h-protein was eluted with the void volume. Ligandin was eluted after the KCl gradient was applied. Ligandin was determined by radial immunodiffusion (24). GSH S-transferase activity: using 1,2-epoxy-3(p-nitrophenoxy)propane as substrate (23), (▲--▲); GSH S-transferase activity using 3,4-dichloro-nitrobenzene (23) as substrate, (◆—◆).

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and azo dye carcinogenesis. On the other hand, binding to the h-protein is specific to the former, while binding to the 4.7S protein is specific to the latter. This raises the possibility that carcinogens may have specific protein receptors (4,29). There are three roles that such a receptor might have. Abell and Heidelberger (4) in 1962 postulated that such a receptor may facilitate the transport of the activated metabolite

of the carcinogen to specific sites in the nucleus. The receptor protein could also function in a detoxification role to facilitate the excretion of the carcinogen. It is also possible theoretically that the carcinogen-protein conjugate could act as an altered derepressor leading to a perpetuated change (30,31).

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