Principal Polypeptide Target of Carcinogen at the Beginning of Liver Carcinogenesis by Three Carcinogens


ABSTRACT

A cytosolic 14,000-dalton polypeptide in rats was previously reported to be the principal target protein of a tracer dose of the carcinogen N-2-fluorenylacetamide in normal liver and liver at the start of hepatocarcinogenesis by three types of carcinogens. Short-term ingestion of any of the carcinogens, the aromatic amide N-2-fluorenylacetamide, the aminoazo dye 3'-methyl-4-dimethylaminoazobenzene, or the amino acid analog ethionine, causes a marked loss of the ability to make the labeled fluorenyl carcinogen:polypeptide complex and a great reduction in the content of the target polypeptide itself. The present report describes the purification, partial characterization, and species and organ distributions of the target polypeptide. A 3-step procedure was devised for the purification of the target polypeptide from normal rat liver cytosol based on the charge and size of the 14C-carcinogen:polypeptide complex from normal rats given N-[9-14C]-2-fluorenylacetamide. The purified target polypeptide was homogeneous according to molecular size and immunoreactivity following sodium dodecyl sulfate:gel electrophoresis. The polypeptide is basic with an isoelectric pH of 8.3. Nevertheless, the polypeptide has 1.6-fold more acidic than basic amino acids, suggestive of the prevalence of amidated glutamic and aspartic residues. The molecule has no detectable tryptophan; one cysteine; two residues of arginine, histidine, tyrosine and proline; and three alanines. An abundance of five residues of methionine and the scarcity of other known potential biological nucleophilic amino acids (zero tryptophan, one cysteine, two tyrosine, two histidine) makes methionine the most likely target of activated carcinogen in the covalent interaction that is apparently involved in the formation of the carcinogen:polypeptide complex. Bound carbohydrate was not detected. Rabbit antisera against the purified target polypeptide reacted specifically with both the polypeptide and its complex with carcinogen. Using sensitive autoradiographic detection of its immunoreactivity, the target polypeptide was found to be present among the liver cytosolic proteins of normal rats, mice, and hamsters of both sexes following sodium dodecyl sulfate:gel electrophoresis, but not in similarly resolved cytosols of their many other organs, indicative that the polypeptide functions mainly (but not exclusively) in liver. Thus far, all of the known principal target proteins of different carcinogens during chemical carcinogenesis are basic proteins in the cytosols of the target organs.

INTRODUCTION

Recent studies dealing with mechanisms of chemical carcinogenesis have focused on early events that occur during the oncogenic process (13, 14). The study of the courses of carcinogens in their interactions with cellular macromolecules in target organs led to the discovery of 2 early events during liver carcinogenesis by 3 types of chemicals (4, 5). Metabolites of a tracer dose of the hepatic carcinogen [9-14C]-FAA in normal liver form a cytosolic 14C-fluorenyl carcinogen:polypeptide complex of small macromolecular size (25; 14,000 daltons). Continual ingestion of any of 3 hepatocarcinogens causes a marked reduction in the ability to make this 14,000-dalton complex and a concurrent increase in the formation of a 14C-fluorenyl carcinogen:protein complex of large molecular weight (7.5S; M, ~150,000). The reciprocal changes in production of the 2 complexes have the following properties. This inversion is brought about by the ingestion of any of 3 hepatocarcinogens, the aromatic amide FAA, the aminoazo dye 3'-Me-DAB, or the amino acid analog ethionine. The alteration occurs early during liver carcinogenesis. Unlike with other reported early events during hepatocarcinogenesis (13, 14), chemical carcinogen appears to be directly involved. The rate of the inversion is associated with the susceptibility of the strain and sex of the rat to the carcinogen; the greater the susceptibility the more rapid is the inversion. The 2 labeled fluorenyl proteins (14,000 daltons and M, ~150,000) are the principal 14C-carcinogen:protein complexes in rat liver cytosol. The loss of the ability to make the 14,000-dalton complex is accompanied by the reduction in the content of the free 14,000-dalton target polypeptide. In contrast to these actions, neither the noncarcinogenic chemical analogs fluorene or aminoazobenzene, nor 3-methylcholanthrene or phenobarbital which induce microsomal monoxygenases, nor the control diets (no carcinogen) cause the inversion in ability to form the 2 complexes. Liver regeneration following partial hepatectomy brings about a moderate inversion in ability to produce the 2 complexes, suggesting that the cell proliferation associated with hepatocarcinogenesis may be causally related to these molecular changes. Consistent with this possibility is the fact that the 14,000-dalton polypeptide has a molecular weight in the range of known polypeptide growth regulators.

Prompted by these unique properties of the 14,000-dalton target polypeptide and of its complex with carcinogen, we have purified the target polypeptide from normal rat liver, character-
ized it in part, and determined its organ and species distributions. These accomplishments have been directed to ascertaining (a) the function of the target polypeptide in normal liver, (b) the biological consequences of the interaction of the polypeptide with carcinogen, and (c) the significance of the subsequent loss of the polypeptide from liver cytosol early during hepatocarcinogenesis by the 3 types of chemicals.

MATERIALS AND METHODS

Animals and Carcinogens

In the purification of the 14,000-dalton target polypeptide (no carcinogen), normal male rats (300 to 385 g) of the Fischer 344 CDF strain (Charles River Breeding Laboratories, Wilmington, Mass.) were maintained ad libitum on a commercial stock diet (Wayne Lab-Blox; Allied Mills, Chicago, Ill.) and acidified tap water.

In isolations of the radioactive 14,000-dalton carcinogen:polypeptide complex, male CDF rats (297 to 308 g) fed the stock diet or female CDF rats (184, 197 g) fed a semisynthetic ethionine control diet (2.4%) of electrofocused protein.

Liver Cytosols

Cytosols were rapidly prepared at 1-4 °C from livers that were perfused portly with 0.25 m sucrose, minced, and homogenized 1:1 (w/v) in 0.25 m sucrose solution using a Potter-Elvehjem Teflon:glass homogenizer (A. H. Thomas Co., Philadelphia, Pa.) (4, 5, 35). The homogenates were centrifuged at 100,000 × g for 1 hr yielding cytosols containing 38 to 50 mg/ml of protein that were stored at −60°C until used.

Purification Procedure of the 14,000-Dalton Target Polypeptide and Its Carcinogen Complex

The 14,000-dalton labeled carcinogen:polyepiprotein complex was first isolated from livers of rats given [9-14C]FAA. The same protocol was thereafter applied to the 14,000-dalton target polypeptide from normal rats (no carcinogen). The procedure consisted of 3 steps.

Gel Filtration (Step 1). Liver cytosol from 2 to 4 normal rats containing 1.0 to 2.5 g of protein (38 to 50 mg/ml) was eluted through a column (200 × 5.3 cm) of Sephadex G-200 gel (Pharmacia Fine Chemicals, Piscataway, N. J.) in 0.01 m Tris-HCl buffer (pH 7.4), and 0.1 m NaCl (4, 5, 35). The isolation of the 14,000-dalton protein was monitored by measurement of protein spectrophotometrically at 235 nm (35), by Lowry assay, and by analytical SDS:polyacrylamide gel electrophoresis. Chart 1 and our previous reports (4, 5, 35, 36) demonstrate typical molecular size profiles containing the 2S component, which was pooled, divided into 50-ml aliquots of protein (6 to 28 mg/ml in different isolations), and stored at −60°. A representative Step 1 that started with 2.2 g of liver cytosolic proteins yielded 336 mg (15%) of 2S proteins.

Preparative Isoelectric Focusing (Step 2). A 100-ml sample of the 2S pool from Step 1 was concentrated to 0.5 volume by Ultrafiltration on a UM-2 membrane (molecular weight cutoff, 1000; Amicon, Lexington, Mass.) and was mixed with an equal volume of 10 m deionized urea. (One liter of 10 m ultrapure urea (Schwarz/Mann, Orangeburg, N. Y.) was deionized at room temperature by stirring for 16 hr with 5 g of AG 501-XBD mixed-bed resin (Bio-Rad Laboratories, Richmond, Calif.), filtered, and kept for up to 1 month at room temperature.) A 2.5-ml sample of the resulting solution containing up to 2.0 mg of protein in 5 m urea was applied to each of 20 cylindrical gels (15 × 1 cm) prepared as described by Righetti and Drysdale (28), using 5% acrylamide, 0.2% methylene bisacrylamide, 6 m deionized urea, and 1% carrier ampholytes at pH 3.5 to 10 (Ampholine; LKB Instruments, Inc., Rockville, Md.). The proteins were focused at 4° for 16 hr at 150 V and then for 1 hr at 380 V, the current decreasing from 5 ma/gel to zero.

One electrophoresed gel was sliced longitudinally into 2 strips. One strip was washed with 10% trichloroacetic acid and stained and destained at room temperature according to the method reported by Maurer (22). The second strip was cut into 5-mm sections, each of which was soaked in 0.5 m of water overnight for pH measurement. The protein bands in the 19 unstained gels were located by use of the reference-stained strip after it was swelled in water to its original length (Fig. 1, Lane IF). Corresponding 3-mm segments were cut, pooled, and successively extracted 3 times by homogenization for 5 min in 15 ml of 0.1% SDS solution in a glass:Teflon-Potter-Elvehjem homogenizer (Arthur H. Thomas Co.; Size B), followed in each case by centrifugation and decantation through Whatman No. 1 paper. Corresponding extracts were pooled and freeze-dried, and the powder was dissolved in 2.5 ml of water. The proteins were precipitated by the addition of an equal volume of acetone and incubation for 3 hr at 4°, centrifuged, and suspended in 0.3 to 0.5 ml of 0.01 m Tris-HCl buffer at pH 7.4 and 0.1 m NaCl. Acetone supernatants containing residual protein were pooled and concentrated, and the proteins were recovered as above.

Analyses of the bound 14C-fluoroenyl distributions in isolations involving labeled carcinogen and analytical polyacrylamide gel electrophoresis in SDS-containing buffer identified the gel extract containing the bulk of the 14,000-dalton carcinogen:polypeptide complex (see "Results"). This protein is indicated by an arrow in Lane IF of Fig. 1. A representative Step 2 that started with 338 mg of 2S proteins yielded 8.1 mg (24%) of electrofocused protein.

Preparative SDS: Gel Electrophoresis (Step 3). Samples of 1.5 mg electrofocused protein from normal rats, 1 m deionized urea, 2% β-mercaptoethanol, 0.02 m Tris-HCl buffer (pH 6.8), and 0.01% pyronin Y protein were immersed in boiling water for 3 min. An aliquot of approximately 150 µg protein was applied to each of 10 wells of a 1-cm stacking gel (3.4% acrylamide;0.1% methylene bisacrylamide) above a resolving gel (2.4%) of electrofocused protein. The proteins were focused at 4° for 16 hr at 40 V (constant) and 10 ma (initially), declining to 5 ma (finally) until the pyronin Y stain reached the bottom of the gel. The track containing the molecular weight standards and a one-half width of an adjacent lane containing sample were excised, stained in 0.05% Coomassie Brilliant Blue R250 in methanol:water:acetic acid (5:5:1), and destained in methanol:water:acetic acid (5:16:1). Molecular weights of the reference proteins were then determined after the gels were shrunken to their original lengths by addition of ethanol to the destain mixture. Fig. 1, Lane Prep, shows a stained track containing the 14,000-dalton polypeptide. This polypeptide was then excised from the unstained gels, extracted, lyophilized, precipitated, and recovered as in Step 2. The product of 6 gels in Step 3, using a total of 8.1 mg of isofocused protein, yielded 2.7 mg (33%) of the polypeptide.

Analytical SDS:Polyacrylamide Gel Electrophoresis

Analytical electrophoreses in 15% polyacrylamide slab gels were carried out as in Step 3 (similar dimensions, except 1.25 mm thick). Samples up to 10 µg protein were electrophoresed for 18 hr at 40 V (constant) and 10 ma initially, decreasing to approximately 5 ma finally. After staining as above, subunit molecular weights were determined.
Chart 1. Molecular size profile of rat liver cytosolic macromolecules, showing the separated 2S class of proteins (Step 1 in the purification of the 14,000-dalton polypeptide). Rat liver cytosol was eluted through a column of Sephadex G-200 gel. Indicated is the 2S region of the profile ("pool") used in the subsequent purification. Details are in the text. (See Chart 2 and Fig. 1, Lane 2S, for the molecular size analysis of the 2S proteins and 2S carcinogen:protein complexes separated by SDS gel electrophoresis.) Related profiles have been reported previously (4, 5).

relative to the standard proteins: phosphorylase b (M, 94,000); bovine serum albumin (M, 68,000); catalase (M, 57,500); ovalbumin (M, 44,000); carbonic anhydrase (M, 28,000); soybean trypsin inhibitor (M, 20,000); and lysozyme (M, 14,300).

Extractions of Carcinogen Derivatives Bound to 14,000-Dalton Polypeptide

As a test of whether the carcinogen binds covalently to the 14,000-dalton polypeptide, a normal male Sprague-Dawley SD rat weighing 440 g was given a dose of [9-¹³C]FAA and sacrificed 48 hr later (see above). The macromolecules in 4 ml of liver cytosol were sieved through a column (196 x 2.5 cm) of Sephadex G-200 gel as in Isolation Step 1, yielding labeled 2S proteins that were stored at -80°C until used (4, 5, 35, 36). After lyophilization, the 2S proteins (2000 cpm; approximately 400 μg) in 1 ml of water were successively extracted twice with 3 ml of methanol:ethyl ether (1:2), 3 times with 95% ethanol, and twice with ethyl ether, by the method of King et al. (19).

Extraction with 14,000-Dalton Target Polypeptide

A solution of the purified 14,000-dalton target polypeptide in 0.15 M NaCl was emulsified with an equal volume of Freund's complete adjuvant (Difco). The emulsion was injected intradermally into New Zealand White rabbits according to the following schedule: 75 μg protein at Week 0; 50 μg at Week 1; 25 μg at Weeks 2, 4, 7, and 9; and 100 μg at Week 15. Precipitating antibodies against the target polypeptide and the carcinogen:polypeptide complex were detected by Ouchterlony double-diffusion immunoprecipitation (26) in agarose banks era/. (11, 12).

Immunization with 14,000-Dalton Target Polypeptide

A solution of the purified 14,000-dalton target polypeptide in 0.15 M NaCl was emulsified with an equal volume of Freund's complete adjuvant (Difco). The emulsion was injected intradermally into New Zealand White rabbits according to the following schedule: 75 μg protein at Week 0; 50 μg at Week 1; 25 μg at Weeks 2, 4, 7, and 9; and 100 μg at Week 15. Precipitating antibodies against the target polypeptide and the carcinogen:polypeptide complex were detected by Ouchterlony double-diffusion immunoprecipitation (26) in agarose gels beginning at Week 5 (not shown). Concentrated solutions of the immunoglobulins (68 mg/ml) were prepared by precipitation of the antisera at 50% saturated ammonium sulfate.

Autoradiographic Immunological Analyses

The autoradiographic immunological analyses of the 14,000-dalton polypeptide used the "western blot" technique (6). After analytical SDS electrophoresis in 15% polyacrylamide gels, the proteins were electrophoretically transferred to nitrocellulose filter paper (BA85-R597, 0.45 μm; Schleicher and Schuell, Inc., Keene, N.H.) in 0.025 M Tris:0.186 M glycine (pH 8.8) and 0.05% SDS using 20 V and 290 ma for 6 hr at room temperature. The nitrocellulose filter paper containing bound proteins was washed for 90 min with 5% bovine serum albumin (radioimmunoassay grade; Sigma Chemical Co., St. Louis, Mo.) in 0.01 M Tris-HCl (pH 7.4) and 0.9% NaCl solution in order to reduce nonspecific protein binding. Following rinsing in the Tris:0.9% NaCl solution buffer (no albumin), the immobilized proteins were reacted for 90 min with the immunoglobulins (diluted 1:43 relative to their protein concentration in antiserum) directed against the 14,000-dalton polypeptide in the above albumin:Tris:0.9% NaCl solution buffer. After 4 washes with the Tris:0.9% NaCl solution buffer (no albumin), 2 washes in 0.05% Nonidet P-40 (Bethesda Research Laboratories, Rockville, Md.) in the Tris:0.9% NaCl solution buffer, and 4 additional washes with the Tris:0.9% NaCl solution buffer, the bound immune complexes were reacted for 90 min with Staphylococcus aureus 125I-Protein A (2 x 10⁶ to 5 x 10⁶ cpm/ml) in the above serum albumin:Tris:0.9% NaCl solution buffer. (The Protein A (Pharmacia) was iodinated with 125I using the solid-phase lactoperoxidase:glucose oxidase system (Enzymobeads; Bio-Rad) (16, 31).) Following further rinsings with the Tris:0.9% NaCl solution buffer, Nonidet P-40 buffer, and Tris:0.9% NaCl solution buffer again, all as above, the immobilized polypeptide:antibody:125I-Protein A complexes were exposed to Kodak XAR-5 film and a Cronex Lighting Plus Intensifier screen (DuPont, Wilmington, Del.) at -80°C. Control experiments used equivalent fractions from corresponding preimmune rabbit sera.

Immunoprecipitation of the ¹⁴C-Carcinogen:Polypeptide Complex

The reaction of the rabbit immunoglobulins with the ¹⁴C-carcinogen complex of the 14,000-dalton polypeptide was examined as one measure of the specificity of the antiserum. A 3.0-ml aliquot of the 2S fraction (0.7 mg/ml) containing ¹⁴C-labeled complex (2721 cpm) from livers of normal male CDF rats was incubated with 2.7 ml of whole globulin fraction (40 mg/ml) from immune or preimmune rabbit sera for 1 hr at room temperature, followed by 4 days at 4°C. (Single levels of immune or preimmune globulins were used because of the lack of plentiful supply. Total precipitation of the complex was thus not attempted.) After addition of 2 ml of Protein A-Sepharose CL-4B gel (Pharmacia) in 0.01 M Tris-HCl buffer, pH 7.4, containing 0.1 M NaCl, incubation for 20 min at room temperature, and centrifugation, the
precipitate was washed 3 times with that buffer. The gel was then suspended in 2 ml of a 24.5 mM Na₂HPO₄:52 mM citric acid buffer, pH 3.0, and heated at 100° for 3 min. After centrifugations, the supernatant fluid and 2 washes of the precipitate in that buffer were pooled and counted in Aquasol 2 in a β-scintillation counter.

Amino Acid Analyses

Purified 14,000-dalton polypeptide from normal male CDF rats was dialyzed against high-purity deionized water in a Spectrapor bag (molecular weight cutoff, M, 3500, 18 mm; Arthur H. Thomas Co.) for 3 days. The protein precipitated and was dissolved in a final concentration of 10 mM sodium phosphate buffer, pH 7.0. Hydrolyses were performed in constant boiling 6 M HCl under reduced pressure for 24, 48, and 72 hr. The 24-hr hydrolysates were derived from one preparation of the purified polypeptide; the 48- and 72-hr hydrolysates were from another preparation. After the hydrolysates were dried, they were analyzed using a D-500 amino acid analyzer (Dionex, Sunnyvale, Calif.). Analysis of each hydrolysate was carried out in duplicate or triplicate. Values for threonine and serine were extrapolated to zero time of hydrolysis. Cysteine and half-cystine were determined as cysteic acid in a performic acid-oxidized sample (24). No significant difference in content of methionine in 6 M HCl hydrolysates was observed with or without prior oxidation with performic acid to methionine sulfone. Tryptophan in a single hydrolysate was assayed in duplicate following digestion of the polypeptide in 3 N mercaptoethanol.

RESULTS

Molecular Size of the Principal Carcinogen:Polypeptide Complex. Administration of a tracer dose of the labeled liver carcinogen [9-14C]FAA to rats fed commercial stock or control diet (no carcinogen) gives rise to the principal labeled carcinogen:protein complex in liver cytosol among the 2S macromolecules (4, 5, 35, 36). The 2S protein fraction contains essentially only one labeled adduct, the principal complex in cytosol with a molecular weight of 14,000, as separated by SDS electrophoresis in 15% polyacrylamide gel (see Fig. 1, Lane 2S, with an arrow at the 14,000-dalton polypeptide). When successive 3-mm sections of preparative SDS:electrophoretic gels were extracted in 0.1% SDS (as in Step 2 of the purification procedure), a total of 79% of the applied radioactivity was recovered. Virtually all (>82%) of the recovered 14C-carcinogen derivatives were bound to a polypeptide with a molecular weight of 13,700 (Chart 2). This molecular weight, determined in under dissociative conditions (SDS, urea, β-mercaptoethanol; "Materials and Methods"), is comparable with the Mₐ value of approximately 10,000 for the average native 2S macromolecule in rat liver cytosol (35). This agreement indicates that the native principal carcinogen:protein complex exists in liver cytosol as a M, 14,000 monomer.

Apparent Covalent Linkage of Carcinogen in the 14,000-Dalton Carcinogen:Polypeptide Complex. The bound 14C-fluorenyl derivatives of the 14,000-dalton complex cannot be released by serial extractions with organic solvents. Thus, 96% of the labeled carcinogen derivatives that are bound to the 2S macromolecules resisted numerous successive extractions with organic solvents of different polarities ("Materials and Methods"). Apparently, the fluorenyl carcinogen is activated to reactive species that combine covalently with the polypeptide prior to and/or after the denaturation attending the solvent extractions.

Purification of the Basic 14,000-Dalton Target Polypeptide and Carcinogen:Polypeptide Complex. The 3-step isolation procedure yielded the 14,000-dalton carcinogen:polypeptide complex in a final state of purity that was indicated by a single protein band and a single labeled complex in SDS electrophoresis in polyacrylamide gel. Parallel preparations of the target polypeptide (no bound carcinogen) showed identical homogeneity.

The efficacy of each step of the isolation procedure was monitored by SDS:gel electrophoresis in terms of 2 parameters, namely, the purity of the protein and the radiochemical homogeneity of the carcinogen:polypeptide complex, both with a molecular size of 14,000 daltons. The purification procedure started with the heterogeneous mixture of proteins and complexes in rat liver cytosol. This protein heterogeneity is shown in Fig. 1 (Lane cyt). The numerous labeled complexes of different molecular sizes present in liver cytosol have been described previously (4, 5, 36). Those reports demonstrated that the principal labeled carcinogen:protein complex generated by labeled FAA in normal or control liver is a 2S macromolecule. Therefore, the first step in the purification of the complex involved the isolation of the 2S proteins from liver cytosol by molecular sieving in Sephadex G-200 gel (Chart 1). The 2S proteins thus obtained had a molecular weight heterogeneity according to SDS gel electrophoresis that is shown in Fig. 1 (Lane 2S). Essentially, the only (>82%) labeled carcinogen-protein complex had a molecular weight of 13,700 (Chart 2 and above). An arrow points to the protein band at this molecular size in an SDS electrophoretic gel (Fig. 1, Lane 2S). When the 2S proteins were subjected to isoelectric focusing in the pH range 3.5 to 10 in 6 M urea, they resolved into many constituents (Fig. 1, Lane IF). The multiplicity of the labeled complexes resolved by this type of separation is shown in Chart...
3. The principal complex was isoelectric at pH 8.3 ± 0.12 (S.D.) (28 determinations). Two less prominent, broadly distributed complexes of approximate isoelectric pH 7.5 and pH 6 are also evident. The principal complex of isoelectric pH 8.3 has a molecular weight of 14,000, as determined by measurement of the bound \(^{14}C\)-carcinogen derivatives resolved in preparative SDS electrophoretic gels (Chart 4). An arrow points to this protein in a stained preparative gel (Fig. 1, Lane prep). Finally, the isolation of the 14,000-dalton complex from the preparative gels yielded a single protein when rerun in analytical SDS:gel electrophoresis (Fig. 1, Lane anal). Occasionally, the product included a small amount of 28,000-dalton protein, possibly the dimer of the polypeptide. A repeat preparative SDS electrophoresis served then to yield a single species.

Amino Acid Composition of the Basic 14,000-Dalton Target Polypeptide. The amino acid composition of the basic 14,000-dalton target polypeptide has several unusual features. As listed in Table 1, there is a 1.6-fold excess of acidic residues compared to basic residues (27 versus 17). The direction and extent of the imbalance are unexpected, in view of the high isoelectric pH (8.3) of the polypeptide. Presumably, many of the acidic residues are amidated. Another noteworthy property is the high content of methionine (5 residues) relative to the levels of the neutral amino acids alanine, leucine, isoleucine, and valine (3, 6, 7, and 9 residues, respectively). The presence of 5 residues of methionine is in marked contrast to the scarcity of the other potential nucleophilic amino acid targets of carcinogens (0 tryptophan, 1 cysteine, 2 tyrosine, and 2 histidine). The single cysteic acid in the hydrolysate is presumably derived from one cysteine. Few aromatic residues are present (2 tyrosine, 5 phenylalanine, 0 tryptophan). The listed quantity of glycine is tentative, inasmuch as the isolation of the polypeptide involved the use of a glycine buffer in SDS:gel electrophoresis, and removal of nonpeptidic glycine by dialysis may not have been complete. The sum of the individual residue weights of the 119 determined amino acids in the polypeptide is \(M, \ 12,794\), in agreement with the molecular weight arrived at by SDS:gel electrophoresis and gel filtration (see above).

Absence of Detectable Carbohydrate in the Basic 14,000-Dalton Target Polypeptide. Bound carbohydrate was not detected in the purified 14,000-dalton polypeptide. SDS electrophoretic gels failed to reveal reaction between the periodic acid-Schiff reagent and the purified polypeptide at levels of 5 to 40 \(\mu\)g. In the same assays, crystalline bovine serum albumin (not a glycoprotein) at quantities of 5 to 20 \(\mu\)g also did not stain for carbohydrate. In contrast, samples of 5 to 20 \(\mu\)g of chicken ovalbumin, which has 3.2\% carbohydrate (43), stained in adjacent electrophoretic lanes. The data indicate that the content of bound carbohydrate in the 14,000-dalton polypeptide is at most 1\%, if not zero.

Specific Immunological Detection of the 14,000-Dalton Target Polypeptide in Liver Cytosol and Its Fractions. The purified 14,000-dalton target polypeptide was homogeneous according to autoradiographic immunoanalyses of preparations resolved by SDS:gel electrophoresis. The isolated polypeptide reacted as a single molecular-size species when exposed to the partially purified immunoglobulins from immunized rabbits (Fig. 2, Lane 14\(K\)). The position of the immunoreactive...
with the immune globulins, and not with the preimmune globulins. Addition of Protein A-Sepharose to the immunoglobulin:2S mixture precipitated 16% of the radioactivity, compared to 1.5% with the preimmune control mixture. [These values included the correction that at least 82% of the 14C of the 2S fraction belongs to the 14,000-dalton complex (see above).] The low level of the 14C immunoprecipitation with Protein A may be explained in part by a weak affinity between the specific antibodies and the conjugated polypeptide or by a hypothetical dissociable noncovalent union of labeled, activated carcinogen metabolite with the undenatured polypeptide (21). Nevertheless, the 10-fold difference in the precipitation of the 14C complex by the immune globulins, compared to the control globulins, is indicative of the specific reactivity of the immunoglobulins against the 14,000-dalton carcinogen:polypeptide complex.

**Immunological Determination of the Organ and Species Distributions of the 14,000-Dalton Polypeptide.** The 14,000-dalton polypeptide was detected only in the livers of rats, mice, and hamsters in analyses of various organ cytosols. In these determinations, 50 or 75 μg of organ cytosolic proteins were resolved by SDS:gel electrophoresis, electrophoretically transferred to nitrocellulose filter paper by the western blot technique, and developed immunologically with 125I-Protein A for autoradiography ("Materials and Methods"). Tests routinely included rat liver cytosols as positive controls. The target polypeptide was not detected in the following organ cytosols in both sexes of the following species: (a) Spd rats, kidney, lung, muscle, heart, stomach, brain, spleen, thymus, pancreas, uterus, mammary gland, testes, and blood; (b) BALB/c mice, kidney, lung, muscle (male), heart, stomach (male), brain, spleen, testes, and blood (male); and (c) Syrian golden hamsters, kidney, lung, muscle, heart, stomach, brain, spleen, pancreas, testes, uterus, blood, and serum. The sensitivity of these analyses was such that the minimal amount of normal rat liver cytosol in which the 14,000-dalton polypeptide was detected was 2.5 μg. Hence, the polypeptide was not detected in the other organ cytosols at levels 20- to 30-fold more than the minimal detection level in liver cytosol. Assuming similar relative concentrations, these other organ cytosols contain less than 3 to 5% of the concentration present in liver cytosol. The detection of the 14,000-dalton target polypeptide in the cytosols of these livers and not of other organs has an important implication (see "Discussion").

**DISCUSSION**

The principal polypeptide target of the metabolites of the hepatocarcinogen FAA in normal rat liver has been purified and partially characterized. The isolated target polypeptide is homogeneous according to molecular size and immunoreactivity following resolution by SDS:gel electrophoresis. The purified target polypeptide has been characterized in terms of its molecular weight, single subunit nature, amino acid composition, isoelectric pH, absence of detectable bound carbohydrate, apparent covalent interaction with carcinogen metabolite in the formation of the principal carcinogen:protein complex of liver cytosol, immunogenicity in rabbits, immunoreactivity on nitrocellulose paper as detected by 125I-Protein A autoradiography, and predominance in liver cytosols and lack of detection in other organ cytosols from rats, mice, and hamsters. Sufficient
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Table 1

Amino acid composition of the basic 14,000-dalton target polypeptide from normal rat liver cytosol

The basic 14,000-dalton target polypeptide was isolated from liver cytosols of normal male CDF rats and hydrolyzed in constant boiling HCl for 24, 48, and 72 hr, as described in the text. The calculations of residues per mol were based on a molecular weight of 13,700.

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<tr>
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<td>1</td>
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<td>Trace</td>
<td>1</td>
<td>0.8</td>
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<tr>
<td>Tryptophan*</td>
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<td>Trace</td>
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<tr>
<td>Total no. of residues</td>
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<tr>
<td>Total residue wt</td>
<td>12,794</td>
<td></td>
<td></td>
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</table>

* Values were extrapolated to zero time of hydrolysis in HCl.

** Inadequate removal of free glycine by dialysis of the 14,000-dalton polypeptide resulted in high levels of this amino acid in the 24-hr hydrolysate. These values were rejected.

a No significant difference in content of methionine was observed in 6 N HCl hydrolysates following prior performic acid oxidation to methionine sulfoxides.

Cysteine was determined as cysteic acid in one analysis of performic acid-oxidized protein (24). The content of cysteine was calculated relative to the amounts of glutamic and valine residues as determined in all 7 HCl hydrolysates.

Tryptophan was not detected in 2 analyses of a single hydrolysate obtained by digestion of the polypeptide in 3 N mercaptoethanesulfonic acid for 24 hr at 110° (27).

information may now be at hand to identify the polypeptide if it has been described previously in another connection or to eliminate the candidacies of known proteins. The present findings thus lay the groundwork for the determination of the functional consequences of the interaction of the target polypeptide with liver carcinogen and of the subsequent loss of the polypeptide from liver cytosol early during liver carcinogenesis.

The 14,000-dalton polypeptide is present in highest concentration in liver. It was detected immunologically only in the liver cytosols of rats, mice, and hamsters of both sexes, and not in their other organ cytosols, despite the use of highly sensitive autoradiography. These results do not exclude the possibility that the target polypeptide exists in significant amounts in cell types that occur in low frequency in nonhepatic organs. Indeed, immunohistochemical analysis indicates that the polypeptide is present in particular cells of various organs. In any case, the preponderance of the 14,000-dalton target polypeptide in liver compared to other organs indicates that this molecule is associated with a function that is mainly characteristic of that organ.

The 14,000-dalton target polypeptide has several notable chemical characteristics. It has a relatively small macromolecular weight and is a basic protein with an isoelectric pH of 8.3. Bound carbohydrate appears to be virtually or totally absent. Notwithstanding that the polypeptide is basic, acidic amino acid residues are in abundance (10 aspartic, 17 glutamic), as is lysine (13 residues). The 1.6-fold excess of acidic over basic amino acids is suggestive of the prevalence of amidated glutamic and aspartic residues. The apparent absence of tryptophan and the low level of cysteine, arginine, histidine, tyrosine, proline, and alanine are particularly noteworthy (1, 2, 2, 2, 2, and 3 residues, respectively). Aromatic acids are present in low amounts (0 tryptophan, 2 tyrosine, and 5 phenylalanine). Methionine is relatively abundant (5 residues), especially compared to the levels of the other neutral amino acids (3 alanine, 6 leucine, 7 isoleucine, and 9 valine). The ratio of methionine to these 4 neutral amino acids is 3-fold higher than in the average protein (0.20 versus 0.06) (10). The 5 residues of methionine are also considerably in excess over the other biologically relevant nucleophilic amino acids (1 cysteine, 2 tyrosine, 0 tryptophan, and 2 histidine). Thus, one-half of the potential nucleophiles in the 14,000-dalton polypeptide are methionine residues. This high content of methionine makes that residue the most likely potential nucleophilic target of the activated FAA in the covalent interaction that is apparently involved in the formation of the carcinogen;polypeptide complex. In addition, the abundance of methionine may in part contribute to the singular susceptibility of the 14,000-dalton polypeptide to activated FAA in vivo. However, cysteine or the other nucleophiles are not excluded as the target amino acid.

The M, 14,000 polypeptide belongs to a group of known basic proteins of that approximate molecular weight. Members of the class include pancreatic RNase, lysozyme, cytochrome c, and the fatty acid-binding protein. Pancreatic RNase has a molecular size of 12,640 daltons (32) and an isoelectric pH of 4.95 (1). RNase also resembles the 14,000-dalton polypeptide in that its activity is considerably decreased early during liver carcinogenesis by aminoazo dyes (7, 8), diethylnitrosamine (9), and W-nitrosomorpholine (41). However, rat and bovine pancreatic RNases (9) as well as lysozyme (9), fatty acid-
binding protein (17, 18, 23, 25, 42), and cytochrome c (9) all have organ distributions, amino acid compositions, or isoelectric pH values that are significantly different from those of the 14,000-dalton target polypeptide. In addition, we have been unable to demonstrate an interaction between the polypeptide antiserum and rat liver cytosolic protein-bound labeled estrone sulfate, a favored ligand of the fatty acid-binding protein of that organ (42).

Basicity and presence in cytosol are the only common characteristics of the 4 known principal target proteins of different chemical carcinogens in organs undergoing carcinogenesis. (a) The principal target protein of the hepatocarcinogenic aminoazo dyes in rat liver cytosol is basic (34, 38, 39) with an isoelectric pH of 8.5 to 9.0 (18). [The minor target protein of the azocarcinogens is slightly more basic (39) with isoelectric pH values of 8.4 and 9.3 and has been termed ligandin (17, 18).] (b) In normal rat liver and in rat liver at the start of hepatocarcinogenesis by FAA, the aminoazo dye 3'-Me-DAB, and ethionine, the principal target protein of metabolites of FAA is a basic cytosolic polypeptide with an isoelectric pH of 8.3 ± 0.12 (this report). (c) The principal target protein of metabolites of FAA in rat liver cytosol at a somewhat later stage of hepatocarcinogenesis by FAA is also basic (37) with an isoelectric pH of 8.4 ± 0.15. This protein is the same principal target protein (7.5S, ~150,000 daltons) that binds metabolites of tracer levels of FAA during liver carcinogenesis not only by FAA but also by the aminoazo dye 3'-Me-DAB and by ethionine (4, 5, 36). (d) The principal polycyclic hydrocarbon carcinogen:protein complexes in cytosols of mouse liver and probably also in skin are basic with isoelectric pH values of 8.05 and 8.6 (29, 30). The association of the common trait of basicity with target proteins of carcinogens has thus been strengthened with each characterization of principal target protein, starting with the first description of a principal carcinogen:protein complex (33), and continuing to the present report. A hypothesis is that the common basicity may facilitate loose interactions between the principal target proteins and cellular nucleic acids, possibly analogous to the tight interactions of the more basic ribosomal proteins with RNA or the more basic histones with DNA. If so, one of the consequences of a reaction between a chemical carcinogen and its principal target protein may be an alteration of the normal association of that target protein with nucleic acids, in turn leading to multiple cellular aberrations.

The study of the courses of carcinogens in their interactions with cellular macromolecules during carcinogenesis has led to the discovery and characterization of the basic 14,000-dalton target polypeptide. The levels of both the target polypeptide and its complex with carcinogen are markedly reduced in liver cytosol early during hepatocarcinogenesis by the aromatic amide FAA, the aminoazo dye 3'-Me-DAB, and the amino acid analog ethionine (4, 5). Future studies will be directed toward the determination of the significance of these early events during liver carcinogenesis by the 3 chemicals.

REFERENCES


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Fig. 1. Analytical distributions in polyacrylamide gels showing the progressive stages in the purification of the 14,000-dalton target polypeptide from rat liver cytosol. The first lane (cyt) shows the many molecular size species of the rat liver cytosolic proteins (40 µg) separated by SDS:polyacrylamide gel electrophoresis. The cytosol was the starting material for Step 1 in the isolation of the 14,000-dalton polypeptide. The second gel (2S) demonstrates the analogous SDS electrophoretic resolution of the 2S proteins (64 µg) prepared by molecular sieving (product of Step 1 in the purification procedure). The third gel (IF) displays the isoelectric focusing distribution of the 2S proteins (262 µg) as resolved in Step 2 of the separation procedure. The fourth gel (prep) shows the molecular size species of proteins (200 µg) in the preparative SDS electrophoresis (Step 3) of the isoelectric-focused fraction. The fifth gel (anal) shows the molecular size distribution obtained by the analytical SDS electrophoresis of the purified 14,000-dalton polypeptide product obtained in Step 3 (10 µg). The last gel (std) shows the SDS electrophoretic resolution of the standard proteins used for molecular weight determination. Arrows, 14,000-dalton polypeptide. Proteins were stained in Coomassie Brilliant Blue R250. Details are provided in the text.

Fig. 2. Specific reaction of the antisemum against the 14,000-dalton polypeptide in normal rat liver cytosol and its fractions after resolution by SDS gel electrophoresis. Samples correspond to those in Fig. 1 and represent the starting mixtures and products in the 3-step isolation of the 14,000-dalton polypeptide. Lane cyt, normal rat liver cytosolic proteins (50 µg) prior to gel filtration in isolation Step 1; Lane 2S, 2S proteins (product of Step 1) prior to isoelectric focusing in Step 2 (50 µg); Lane IF, product of isoelectric focusing (Step 2) that served as starting material for preparative gel electrophoresis in Step 3 (50 µg); Lane 14K, final purified 14,000-dalton polypeptide (10 µg) that resulted from Step 3. The polypeptides in all lanes were separated by SDS:gel electrophoresis (see Fig. 1), transferred electrophoretically to nitrocellulose filter paper, and reacted with immune globulins (68 mg/ml) against the 14,000-dalton polypeptide and then 125I-Protein A for autoradiography. Only the 14,000-dalton polypeptide in each lane reacted with the antisemum. Details are provided in the text.
Principal Polypeptide Target of Carcinogen at the Beginning of Liver Carcinogenesis by Three Carcinogens


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