Identification of Cytosolic and Microsomal Bile Acid-binding Proteins in Rat Ileal Enterocytes*

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Studies were performed to determine the subcellular fractions and proteins involved in the intracellular transport of bile acids in rat ileal cells. The photolabile derivative 7,7-azo-taurocholate inhibited the Na⁺-dependent uptake of taurocholate into rat ileal enterocytes reversibly in the dark and irreversibly following photolysis. When photolabeled cells were submitted to subcellular fractionation, greatest radioactivity was found in the soluble protein (SP) fraction with decreasing radioactivity in the brush-border- (BBM), basolateral- (BLM), microsome- (MC), and Golgi- (GO) enriched fractions. Following trichloroacetic acid precipitation, delipidation, and correction for loss of marker enzyme activity, protein bound radioactivity was in SP > BBM > MC > BLM > GO > MT. When photolabeled cells were first fractionated and then submitted to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, a 99-kDa polypeptide was associated with BBM, 54- and 59-kDa polypeptides with BLM, 14-, 35-, 43-, 59-, and 6%kDa polypeptides with SP and a 20-kDa polypeptide with MC anti-intestinal fatty acid-binding proteins. No precipitate to the hepatic cytosolic bile acid-binding protein. kDa microsomal, and 14- and 35-kDa cytosolic bile acid-binding polypeptides which may be involved in the active transport process in circulation which involves their synthesis from cholesterol in enterocytes via anion-exchange across the basolateral membranes (12) of the intestine. These intestinal studies demonstrated that the photolabile derivatives shared transporters with natural bile acid and identified a 99-kDa polypeptide and 54- and 59-kDa polypeptides which may be involved in bile acid transport across brush-border and basolateral membranes of the ileal epithelial cell, respectively.

In contrast to our knowledge regarding bile acid transport across the intestinal plasma membranes, relatively little is known regarding those events involved in the intracellular movement across the enterocyte. A distinct advantage of photoaffinity labeling is that a covalent linkage between the photoprobe and binding protein remains intact during cell processing. This advantage has been exploited in the present studies which involve the photoaffinity labeling of enterocytes followed by cell fractionation to localize the subcellular components and putative proteins involved in bile acid transport.

EXPERIMENTAL PROCEDURES

Preparation of Ileal Epithelial Cells—Enterocytes from rat ileum were prepared according to the method of Pinkus (13) as modified in our laboratory (12). The intact enterocytes were centrifuged at 500 × g in a refrigerated centrifuge, and the pelleted cells were washed in preincubation media selected for transport, photolysis, or fractionation experiments. Cell viability was assessed by exclusion of 0.3% trypan blue dye. Only those preparations with >85% viable cells were used for study. In addition, oxygen consumption rates that were recorded polarographically on random preparations were similar to those reported by Pinkus (13). Transport Measurements—Uptake of radiolabeled substrate by ileal epithelial cells was determined by the membrane filtration technique (14). Typically, the transport reaction was initiated by adding 20 µl of preincubated cells to 100 µl of incubation buffer kept in a water bath at 37 °C. The compositions of the preincubation and incubation media are given in the legends to the figures. At desired time intervals, the transport reaction was terminated by the addition of 1 ml of ice-cold stop solution that had the same composition as the incubation medium. Absolute solute uptake was calculated and is expressed as pmol/mg protein. All experiments were performed in triplicate with freshly prepared cells on 2 or more separate days. Analysis of data for significant differences (p < 0.05) was according to the Student's t test for unpaired data (15).

Photoaffinity Labeling—Ileal cells were preincubated in solution A containing (in mM): 89.9 mannitol, 100 NaCl, 10 HEPES/Tris, pH 7.4. The preloaded cells were incubated under red lighting at 25 °C for 1.5–2 min in solution A containing tracer quantities of radiola-
beled photoprobe, yielding final concentrations of 2.0–2.5 mg of cell protein/ml and 4–10 μM [3H]7,7-azo-TC.1 Photoaffinity labeling was carried out with a total volume of 1.4 ml at 25 °C in a cylindrical cuvette made of Pyrex glass (Wilmad Glass Co., Buena, NJ) situated inside a Rayonet photoreactor RPR 190 (Southern New England Ultraviolet Co., Hamden, CT) equipped with 16 symmetrically arranged 350-nm lamps. After photoaffinity labeling, the cells were collected from the cuvette and washed three times by dilution in solution A and centrifugation at 500 × g for 10 min to form a dark pellet (nuclei and mitochondria), top fluffy layer, and supernatant fractions. The top fluffy layer and 200,000 × g pellet were resuspended with a total volume of 1.4 ml at 25 °C in a cylindrical cuvette made of Pyrex glass (Wilmad Glass Co., Buena, NJ) situated inside a Rayonet photoreactor RPR 190 (Southern New England Ultraviolet Co., Hamden, CT) equipped with 16 symmetrically arranged 350-nm lamps. After photoaffinity labeling, the cells were collected from the cuvette and washed three times by dilution in solution A and centrifugation at 500 × g for 5 min.

**Pulse Chase Experiments**—For pulse studies, 250 ml of Krebs bicarbonate buffer, pH 7.4, containing 10 μM (20 μCi) [3H]7,7-azo-TC was added under red lighting to each well (16-mm diameter) of a plastic tray (Corning Glass Works, Corning, NY). Then 250 ml of ileal cell suspension was added to each well (final concentration, 2–3 mg of cell protein/ml) in a timed sequence (7 s ±5 min) prior to photolysis using a 1500 watt xenon flash tube supplied with flash trigger electronics (EIR Electronics, Inc., Charlottesville, VA). For chase studies, cells preincubated for 120 s with 10 μM [3H]7,7-azo-TC were incubated with taurocholate (final concentration, 0.5 mM taurocholate) from 0 to 120 s prior to flash photolysis. After photoaffinity labeling, the cells were collected from the wells and washed twice in ice-cold Krebs bicarbonate buffer.

**Subcellular Fractionation**—Washed, photolyzed ileal cells were suspended in ice-cold isolation buffer (2.5 mg protein/ml) consisting of 5% sorbitol, 0.5 mM EDTA, 5 mM histidine-imidazole, pH 7.4, 0.05% soybean trypsin inhibitor, 0.25 mM phenylmethylsulfonyl fluoride, and 2 units/ml aprotinin. As outlined in Fig. 1, the suspended cells were disrupted in a Parr bomb (Parr Instrument Co., Moline, IL) under 800 psi at 0 °C for 1 min/ml of sample. The cell homogenate was diluted to three times its original volume with isolation buffer and centrifuged at 8,000 × g for 10 min to form a dark pellet (nuclei and mitochondria), top fluffy layer, and supernatant fractions. The supernatant was centrifuged at 200,000 × g for 60 min. The 200,000 × g supernatant was regarded as the soluble protein fraction (100 μg of protein) was mixed gently with 20 μl of rabbit antiserum. After an overnight incubation at 4 °C, 40 μl of 10% heat-killed protein A-Staphylococcus aureus (10 μg of protein) was added, and the mixture was incubated for 30 min at 22 °C under gentle shaking. Following centrifugation at 15,000 × g for 90 min, the precipitate was set aside, and the supernatant was incubated with 30 μl of [3H]7,7-azo-TC at 24 °C for 5 min in the dark. Following centrifugation at 15,000 × g for 5 min, the pellet was washed once in 290 mM sodium phosphate buffer, pH 7.4, and both washed pellet and supernatant were subjected to SDS-PAGE.

**Intracellular Intestinal Bile Acid-binding Proteins**

1 The abbreviations used are: 7,7-azo-TC, 7,7-azo-taurocholate; SP, soluble protein; BBM, brush-border membrane; BLM, basolateral membrane; M1, mitochondria; MC, microsome; GO, Golgi; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; L-BABP, liver bile acid-binding protein; L-FABP, intestinal fatty acid-binding protein; L-FABP, liver fatty acid-binding protein.
with the sodium salt of (7,7-azo-3α-12α-dihydroxy-5β-[3H]cholan-
24-oyl)-2-aminoethanesulfonate having a specific radioactivity of 1.25
Ci/mmol or greater (average specific activity = 3.75 Ci/mmol). The
synthesis and properties of this photolabile bile acid derivative have
been previously described (22). [3H]taurocholic acid (6.8 Ci/mmol)
and Protosol were purchased from Du Pont-New England Nuclear.
Taurocholate was purchased from Steraloids, Inc. (Wilton, NH). The
radioactivity of the labeled bile acids was found to be greater than 95% 
using thin layer chromatography. For immunoblotting and radioim-
munoprecipitation, rabbit anti-rat albumin IgG and anti-chicken actin
antiserum were purchased from Cappel (West Chester, PA) and ICN
(Lisle, IL), respectively. Rabbit anti-rat liver and intestinal fatty
acid-binding protein and anti-rat liver cytosolic bile acid-binding
protein were kindly provided by Drs. Robert K. Ockner and Nathan
Bass, University of California Medical Center, San Francisco, CA,
and Drs. Neil Kaplowitz and Andrew Stolz, Wadsworth VA Hospital,
Los Angeles, CA, respectively.

RESULTS

Interaction of 7,7-Azo-TC with Taurocholate Uptake by Ileal
Cells under Red Lighting—In order to identify putative trans-
port proteins by photoaffinity labeling, it first must be demon-
strated that the photolabile derivative can share the transport
system with natural substrates (23). The addition of Na+
ton the incubation media was shown previously to stimulate
bile acid uptake by isolated ileal cells (24). Therefore, the
uptake of 7,7-azo-TC into ileal cells was studied in the pres-
ence and absence of Na+. Fig. 2A shows the effect of Na+ and
taurocholate on [3H]7,7-azo-TC uptake by ileal cells under
red lighting. The presence of sodium instead of choline in the
incubation media resulted in the stimulation of 0.1 mM
7,7-azo-TC uptake. In addition, sodium-dependent 7,7-azo-TC
uptake was inhibited by 0.25 mM taurocholate, whereas up-
take of 7,7-azo-TC in the absence of sodium was unaffected
by the presence of taurocholate in the incubation media.
Conversely, Fig. 2B shows the interaction of 7,7-azo-TC with
the uptake of taurocholate by ileal cells. The uptake of 0.1
mM taurocholate was stimulated by the presence of Na+
in the incubation media. The Na+-dependent uptake of tauro-
cholate was completely abolished by the presence of 0.5 mM
7,7-azo-TC, whereas 7,7-azo-TC had no effect on taurocholate
uptake in the absence of Na+.

Inhibition of Taurocholate Uptake in Ileal Cells by Photo-
affinity Labeling with 7,7-Azo-TC—To test whether the inhi-
bition of taurocholate uptake by 7,7-azo-TC was reversible
without photolysis, the ileal cells were preincubated with or
without 0.5 mM 7,7-azo-TC for 10 min under red lighting.
After washing the cells free of 7,7-azo-TC, the uptake of 0.1
mM taurocholate was measured in the presence or absence of
Na+ in the incubation media. As shown in Fig. 3 (bars 1 and
2), no significant difference in taurocholate uptake was seen
between cells preincubated without or with the photolabile
derivative. This experiment demonstrated that the inhibition
by 7,7-azo-TC under subdued light was completely reversible.
The next experiments were performed to determine whether photolysis of cells in the presence of the photoaffinity probe
would result in an irreversible inhibition of taurocholate up-
take. Irradiation of the cells for 10 min in the absence of 7,7-
azo-TC had no effect on subsequent uptake of taurocholate
(Fig. 3, bar 3) when compared with non-irradiated cells (Fig.
3, bar 1). However, if ileal cells were exposed to UV light for
10 min in the presence of 0.5 mM 7,7-azo-TC, the subsequent
uptake of Na+-dependent taurocholate was significantly re-
duced (Fig. 3, bar 4). Photolaffinity labeling under all condi-
tions had no effect on taurocholate uptake in the absence of
Na+. These studies suggest that photoreacted 7,7-azo-TC
irreversibly inhibited the Na+-dependent bile acid uptake system in ileal cells.

Distribution of Marker Enzyme Activities—After ileal cells
were photolysed in the presence of 7,7-azo-TC and disrupted
in the Parr bomb, the resulting organelles, membrane frag-
ments, and cytosol were fractionated by differential centri-
fugation and sorbitol density gradient separation (Fig. 1).
Marker enzyme activities were used to measure the relative
enrichment of the subcellular fractions. Galactosyl transfer-
ase, K+-stimulated p-nitrophenyl phosphatase, alkaline phos-
phatase and NADPH-cytochrome c reductase were used as
marker enzymes for Golgi (GO), basolateral membranes
(BLM), brush-border membranes (BBM), and microsomes
(MC), respectively. Specific activities of these enzymes
were measured in the differential centrifugation and density gra-
dient fractions. The marker enzyme distribution of these
fractions are summarized in Table I. With respect to starting
cell homogenate, the relative enrichment of 12-fold for galac-
tosyl transferase in the 20/30% sorbitol fraction identified this fraction as being relatively enriched in Golgi membranes. The specific activities of K+-stimulated p-nitrophenyl phosphatase and NADPH-cytochrome c reductase were negatively enriched in this fraction. The relative enrichment of 9.4-fold over starting cell homogenate for K+-stimulated p-nitrophenyl phosphatase in the 30/40% sorbitol fraction indicated that this fraction was relatively enriched in basolateral membranes. The specific activities of Golgi, brush-border membrane, and microsomal enzymes were enriched 3.5-, 0.4-, and 1.0-fold, respectively. The 40/50% and 50% sorbitol fractions contained relatively enriched specific activities for alkaline phosphatase and NADPH-cytochrome c reductase. Magnesium precipitation was used to separate the brush-border membrane- and microsomal membrane-enriched fractions. As shown in Table I, the addition of 10 mM magnesium resulted in a 7-fold enrichment in the alkaline phosphatase and 5-fold enrichment in the NADPH cytochrome c reductase activities in the supernatant and pelleted fractions, respectively. With the exception of galactosyl transferase in the supernatant fraction, the specific activities of the marker enzymes were negatively enriched. The specific activities of the four marker enzymes also were not enriched with respect to starting cell homogenate in the mitochondrial (MT) and soluble protein (SP) fractions. Finally, the activities of the marker enzymes were not enriched in the 50/60%, 60%, and pelleted sorbitol fractions (data not shown).

Distribution of Radioactivity—The intracellular distribution of [3H]7,7-azo-TC was assessed initially by measuring the total amount of radioactivity associated with each enriched subcellular fraction. Eighty-two% of the radioactivity in the cell homogenate was accounted for in the subcellular fractions; the remaining radioactivity presumably was in the uncleaved sorbitol gradient fractions. As shown in Table II, column 2, the majority of the radioactivity (70.7%) was found in the 200,000 × g supernatant (cytosolic or soluble protein fraction). To determine the amount of radioactivity that was protein-bound, each fraction was incubated with 10% trichloroacetic acid at 4°C overnight. The reaction mixtures were filtered through 0.45-μm pore size cellulose nitrate filters and washed with 5 ml of 5% trichloroacetic acid, and the amount of radioactivity remaining on the filters was counted. Table II (compare columns 2 and 3) shows that 37% of the total radioactivity in the cell homogenate fraction was found in the trichloroacetic acid-precipitable cell homogenate fraction. The failure to account for all cell homogenate total radioactivity in the trichloroacetic acid-precipitable cell homogenate may be explained by incomplete photoysis of 7,7-azo-TC. To test for this possibility, 10-μl aliquots of cell homogenate were submitted to thin layer chromatography using the solvent system n-butanol/acetic acid/water (9:2:1, by volume) and cochromatographed with unphotolyzed and photolyzed 7,7-azo-TC as standards. After the plates were developed in the dark, 53.8 ± 1.84% (n = 4) of the radioactivity had an Rf value identical to that of photolyzed 7,7-azo-TC, and the remainder of radioactivity was found at the origin of the plates. Inasmuch as cell homogenate is not expected to run in the solvent system, these observations suggested that photolyzed 7,7-azo-TC was bound to membrane protein which remained at the origin.

To reduce the likelihood of nonspecific partitioning into membrane lipid, filters containing the trichloroacetic acid precipitated fraction were further washed with 5 ml of chloroform/ethanol (2:1, by vol), and the radioactivity remaining on the filters was counted. As shown in Table II, column 4, this extraction step resulted in little reduction of radioactivity for each fraction. The majority of radioactivity found in the TCA-precipitated, lipid-extracted cell homogenate was accounted for in the subcellular fractions with decreasing percentage of radioactivity in these fractions as follows: SP, 32.3%; BBM, 12.2%; BLM, 4.6%; MC, 3.9%; GO, 0.7%. As shown in Table II, column 5, when the percentage of radioactivity was corrected for differences in radioactivity due to incomplete enrichment of the subcellular fractions (i.e. the percentage of radioactivity was divided by the percent yield of marker enzyme for each fraction), the values were as follows: SP, 43.2%; BBM, 30.6%; MC, 27.3%; BLM, 13.1%; GO, 2.2%.
Intracellular Intestinal Bile Acid-binding Proteins

TABLE II

Cellular distribution of [3H]7,7-azo taurocholate after photoaffinity labeling

| Fraction          | Total radioactivity (cpm x 10^6) | Trichloroacetic acid-precipitable radioactivity (cpm x 10^4) | Trichloroacetic acid-precipitable, delipidated radioactivity (cpm x 10^4) | %*      |
|-------------------|----------------------------------|-------------------------------------------------------------|----------------------------------------------------------------------------|---------|
| Cell homogenate   | 212.3 ± 8.0 (100)                | 78.8 ± 1.7 (100)                                            | 53.9 ± 5.4 (100)                                                         | 100     |
| Mitochondria      | 4.1 ± 1.2 (1.9)                  | 4.0 ± 1.1 (5.1)                                             |                                                                            |         |
| Soluble proteins  | 150.2 ± 5.0 (70.7)               | 19.2 ± 1.9 (24.4)                                          | 17.4 ± 1.4 (32.3)                                                        | 43.2    |
| Golgi             | 0.8 ± 0.1 (0.4)                  | 0.6 ± 0.1 (0.5)                                            | 0.4 ± 0.01 (0.7)                                                        | 2.2     |
| Basolateral       | 6.3 ± 0.4 (2.1)                  | 3.8 ± 0.5 (4.8)                                            | 2.5 ± 0.2 (4.6)                                                         | 13.1    |
| Brush-border      | 11.8 ± 0.4 (5.6)                 | 7.7 ± 0.3 (9.8)                                            | 6.6 ± 1.7 (12.3)                                                        | 39.8    |
| Microsomes        | 3.0 ± 0.5 (1.4)                  | 2.4 ± 0.1 (3.0)                                            | 2.1 ± 0.4 (3.9)                                                         | 27.3    |

* % equals radioactivity in trichloroacetic acid-precipitable, delipidated fraction divided by total radioactivity in cell homogenate and the percent yield (Y%) of corresponding marker enzyme activity.

Additional studies addressed the question of specific covalent labeling of subcellular fractions by 7,7-azo-TC. Ileal cells were exposed to [3H]7,7-azo-TC in the presence of UV light with 0 or 0.5 mM taurocholate or absence of both UV light and taurocholate. Following these incubations the ileal cells were fractionated, and the subcellular fractions were precipitated with 10% trichloroacetic acid and extracted with chloroform/methanol. Specific radioactivities were calculated as the bound radioactivity divided by the amount of protein in each fraction. The columns show the means ± S.E. for three preparations. HO, homogenate.

Identification of Bile Acid-binding Polypeptides—For these studies, ileal enterocytes (0.2 mg of protein) were incubated with 10 µCi of [3H]7,7-azo-TC and exposed to UV light for 5 min. After photolysis the cells were washed with HEPES/Tris buffer and then extracted with ethanol/chloroform. The cell proteins were solubilized in SDS buffer and subjected to gel electrophoresis. As shown in Fig. 5, photoaffinity labeling of ileal cells resulted in the incorporation of radioactivity into several polypeptides. The apparent molecular weights of the clearly labeled polypeptides were 99,000, 68,000, 59,000, 54,000, 43,000, 35,000, 20,000, and 14,000. Fig. 5 also demonstrates that the presence of 0.5 and 1.0 mM taurocholate during photoaffinity labeling progressively inhibited the incorporation of radioactivity, suggesting the photolabile derivative is bound to the same polypeptides as natural bile acid.

The next series of studies were performed to determine the intracellular location of bile acid-binding polypeptides. Ileal cells that were photolyzed in the presence of [3H]7,7-azo-TC, were submitted to fractionation as outlined in Fig. 1. Each subcellular fraction was submitted to SDS-PAGE, and the amount of radioactivity incorporated into the separated polypeptides was determined. Employing this approach previously, the 99-kDa bile acid-binding polypeptide was found in the brush border membrane fraction, whereas the 54- and 59-kDa polypeptides were associated with basolateral membranes.

Fig. 4. Distribution of [3H]7,7-azo-TC in subcellular fractions. Ileal cells were exposed to [3H]7,7-azo-TC in the presence of UV light with 0 (closed bars) or 0.5 mM (hatched bars) taurocholate, or in the absence of both UV light and taurocholate (open bars). The ileal cells were fractionated, and the subcellular fractions were precipitated with 10% trichloroacetic acid and extracted with chloroform/methanol. Specific radioactivities were calculated as the bound radioactivity divided by the amount of protein in each fraction. The columns show the means ± S.E. for two experiments performed in triplicate. Values in parentheses indicate the specific radioactivities by upward of 50%, suggesting the values encountered when each fraction was obtained from natural bile acid competed with [3H]7,7-azo-TC for the protein-binding site or a proximal transport step. Finally, incubation of ileal cells with 7,7-azo-TC under subdued lighting resulted in specific radioactivities that were <10% of those values encountered when each fraction was obtained from cells that were photolyzed with the photoprobe.

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Intracellular Intestinal Bile Acid-binding Proteins

Fig. 6. Distribution of radioactivity after SDS-PAGE of soluble protein (A) and microsomal (B) fractions isolated from ileal cells irradiated with [3H]7,7-azo-TC. Cells were irradiated and fractionated as outlined in Fig. 1. The subcellular fraction was subjected to SDS-PAGE. The top solid line indicates the densitometer scanning of the polypeptides after staining, whereas the bottom circled lines indicate the distribution of radioactivity following photolysis of cells in the absence (○) or presence (□) of 0.5 mM TC.

(12). Similar results were again obtained in the presence studies (data not shown), leaving unaccounted the 68-, 43-, 36-, 20-, and 14-kDa polypeptides. Fig. 6A shows the photoaffinity labeling pattern of the soluble protein fraction. Greatest incorporation of radioactivity was seen in polypeptides of 68,000, 59,000, 43,000, 35,000, and 14,000 molecular weight (Fig. 6A, solid line, closed circles). In order to gain further evidence that the photolabile derivative was bound to the same polypeptide as natural bile acid, photoaffinity labeling of ileal enterocytes was performed in the presence of 0.5 mM taurocholate. As shown in Fig. 6A (solid line, open circles), taurocholate selectively inhibited photoaffinity labeling of these polypeptides. Fig. 6B shows the pattern of incorporation of radioactivity into polypeptides of the microsomal fraction that was obtained from ileal cells irradiated with [3H]7,7-azo-TC. As shown in Fig. 6B (solid line, closed circles), greatest incorporation of radioactivity was seen in a 20-kDa polypeptide. Fig. 6B (solid line, open circles) also shows that taurocholate inhibited the labeling of this polypeptide.

To further confirm which proteins have specific binding, direct photoaffinity labeling of the cytosolic fraction was performed. The soluble protein fraction was first obtained from non-irradiated ileal cells as outlined in Fig. 1, then photolyzed in the presence of [3H]7,7-azo-TC, followed by SDS-PAGE. The greatest incorporation of radioactivity was seen again in polypeptides of 68,000, 59,000, 43,000, 35,000 and 14,000 molecular weight (Fig. 7, solid line, closed circles). When photoaffinity labeling of the soluble protein fraction was carried out in the presence of 0.5 mM taurocholate, the bile acid selectively inhibited the labeling of the 68,000, 43,000, 35,000, and 14,000 molecular weight polypeptides (Fig. 7, solid line, open circles).

Pulse-chase experiments were conducted to determine the photoaffinity labeling of bile acid-binding polypeptides with respect to time. Ileal cells were pulsed by incubation with [3H]
Intracellular Intestinal Bile Acid-binding Proteins

B.

20 14kDa

0 sec.

- 7 sec.

- 120 sec.

- 0 sec.

- 60 sec.

- 120 sec.

Migration Distance (cm)

FIG. 8. Time-dependent incorporation of radioactivity into bile acid-binding polypeptides and chase of the labeling with taurocholate. The cells were pulsed (A) with [3H]7,7-azo-TC for 0 ( ), 7 ( ), 60 ( ), and 120 ( ) s, and chased (B) with 0.5 mM TC for 0 ( ), 60 ( ), and 120 ( ) s prior to flash photolysis as described under "Experimental Procedures." The cells were subjected to SDS-PAGE, and the amount of radioactivity incorporated into bile acid-binding polypeptides was determined with respect to time.

7,7-azo-TC prior to flash photolysis. The photolyzed cells were fractionated, each subcellular fraction was submitted to SDS-PAGE, and the amount of radioactivity incorporated into the separated polypeptides was determined. Fig. 8A shows the incorporation of radioactivity following 0-, 7-, 60-, and 120-s pulses. The incorporation of radioactivity at 90 s and 5 min (data not shown) was identical to that obtained at 120 s. In addition, cells preincubated for 120 s with [3H]7,7-azo-TC were chased by incubation with 0.5 mM taurocholate for 0–120 s prior to flash photolysis. Fig. 8B indicates that the taurocholate chase resulted in a progressive decline in photolabeling of polypeptides suggesting the natural bile acid competes with photoprobe for protein-binding sites.

The similarity of molecular masses suggested that the 35- and 14-kDa polypeptides were the rat 33-kDa liver bile acid-binding protein (L-BABP) and the 14-kDa rat intestinal fatty acid-binding protein (I-FABP), or rat liver fatty acid-binding protein (L-FABP), respectively (25, 26). Therefore, these cytosolic bile acid-binding proteins were characterized by immunoblotting. As shown in Fig. 9, polypeptides that were labeled with [3H]7,7-azo-TC, were transferred from SDS gels to nitrocellulose membrane and cut into strips. The strips were incubated with rabbit antiserum against rat L-BABP (A), L-FABP (B), and I-FABP (C). Strip D was cut into 2-mm slices, and the radioactivity was counted in each slice. When strip D was carefully aligned with strips A–C, the migration distance of the radioactive peaks corresponded to that of the blot for L-FABP but not to those for L-BABP or I-FABP.

The identity of the bile acid-binding polypeptides was further explored using radioimmunoprecipitation. Fig. 10 shows that immunoprecipitation experiments were performed with [3H]7,7-azo-TC-labeled cytosolic polypeptides using rabbit
Identification of cytosolic bile acid-binding polypeptides by radioimmunoprecipitation.

Polypeptides of the solubilized protein fraction, labeled by [3H]7,7-azo-TC, were incubated with rabbit antisera against albumin (B), actin (C), L-BABP (E), I-FABP (F), and L-FABP (G). The precipitates and untreated cytosolic polypeptides (A and D) were subjected to SDS-PAGE. The solid lines indicated the distribution of radioactivity in the sliced gels.

Antisera against albumin (B), actin (C), L-BABP (E), I-FABP (F), and L-FABP (G). The precipitates and untreated cytosolic proteins (Fig. 10, A and D) were subjected to SDS-PAGE at 9% (A–C) and 11% (D–G) polyacrylamide. When the gels were sliced and counted for radioactivity, radioimmunoprecipitation was noted with antisera against albumin and actin but not with anti-L-BABP, anti-L-FABP, or anti-I-FABP.

The failure to note immunoprecipitation against rabbit antisera may be explained by the masking of antigenic sites due to covalent linkage between photoprobe and binding protein. To test this possibility cytosolic protein was first incubated with rabbit antiserum adsorbed to protein A-Sepharose. Following centrifugation the supernatant was photolabeled with [3H]7,7-azo-TC and subjected to SDS-PAGE. The amount of radioactivity incorporated into bile acid-binding protein in the supernatant was compared with the amount of radioactivity incorporated into bile acid-binding protein that was not incubated with antibody (control). The incubation of cytosolic protein with rabbit anti-rat L-FABP did not result in loss of radioactivity when compared with control (cf. Fig. 11, D with E).

DISCUSSION

A criteria for the identification of transport proteins using photoaffinity labeling techniques is that upon photolysis the photoprobe irreversibly inhibits the transport system (23). In previous studies from our laboratory (12), ileal cells were preincubated with [3H]taurocholate in the presence of 7,7-azo-TC under subdued or UV lighting, and the efflux of taurocholate was measured after adding the cells to media free of bile acid. The irradiation of cells with UV light in the presence of the photolabile derivative inhibited the subsequent efflux of taurocholate to a significantly greater degree than preincubation in the presence of 7,7-azo-TC under red lighting, suggesting an irreversible biochemical modification of the bile acid exit system by the photoactivated derivative (12). In the present study the unreacted photolabile derivative interacted with the cellular uptake of bile acid via the brush-border Na+-dependent transport system, and the reacted photoprobe inhibited irreversibly bile acid uptake. Taken together, these observations indicate that 7,7-azo-TC shares and irreversibly inhibits both uptake and exit transport systems and appears well-suited for the identification of the putative intracellular transport polypeptides for bile acids.

A number of experiments were performed to further validate the significance of individual fractions and binding proteins being identified. First, the photolysis of enterocytes with [3H]7,7-azo-TC demonstrated reproducible labeling patterns when performed repeatedly under identical experimental conditions. Second, experiments were carried out under identical conditions except that taurocholate was added during photolysis. The partial inhibition of incorporation of radioactivity from [3H]7,7-azo-TC shares and irreversibly inhibits both uptake and exit transport systems and appears well-suited for the identification of the putative intracellular transport polypeptides for bile acids.

A high intensity UV photoreactor and a specially designed glass cuvette were used to provide greatest exposure of light to individual cells in suspension (4). Despite these efforts, significant losses of radioactivity occurred during the washing and extraction of the cell homogenate. When the radioactivity in the cell homogenate was submitted to thin layer chroma-
protein were incubated in the absence and presence of anti-L-FABP.
FABP was centrifuged. The supernatant fraction and cytosolic pro-
gels B and C, respectively.
be assessed quantitatively. Moreover, experiments, performed
under conditions identical for photolysis but in the absence
of hepatic cytosolic bile acid binding proteins with different
molecular masses have been identified: (a) glutathione S-
transferase (45–50 kDa), (b) Y’ binders (33 kDa), and (c)
fatty acid binding proteins (FABP) (14 kDa). These cytosolic
proteins may facilitate vectorial bile acid transport by mini-
mizing back diffusion from the cell and retaining bile acids
within the cytosolic compartment, thereby preventing distribu-
tion into the membranous compartments (35). It is also
conceivable that directionality of transport is maintained
should the binding proteins have specific membrane affinity
sites at the pole of the cell opposite from where bile acids are
initially taken up. The association of the 59-kDa polypeptide
with both the soluble protein and basolateral membrane frac-
tions in the present studies suggests this latter possibility.
The similarity of molecular masses suggested that the 35-
and 14-kDa bile acid-binding polypeptides, identified by
photoaffinity labeling, were Y binder and FABP, respectively.
The presence of intestinal proteases has prevented an accu-
rate measurement of Y binders in the small intestine, al-
though a 33-kDa protein band was identified on immunoblot-
ting of small intestinal cytosol (35). In contrast, the rat small
intestinal epithelium has been shown to contain two abundant
FABPs. The L-FABP, present in both liver and intestine, is
a polypeptide of 14,184 which is identical to Z protein; the L-
FABP, present in the intestine, is a 15,063-Da protein and represents 2% of the soluble intestinal proteins (36). Studies have supported a role for FABP in intestinal fatty acid uptake, intracellular transport and utilization, but the question remains whether or not FABP serves a broader function in the intracellular handling of amphipathic small molecules such as bile acids. Bile acids are capable of binding to the L-FABP (37). However, the lower binding affinity of bile acids to L-FABP than the affinity of long chain fatty acids has argued against an important role for L-FABP in bile acid intracellular transport (35). In the present studies, the specific antibodies to L-BABP, I-FABP, and L-FABP recognized on immunoblotting 33- and 14-kDa polypeptides, respectively. Except for L-FABP, the blotted protein bands had a different migration distance than because immunoprecipitation of cytosolic protein did not reduce the subsequent photoaffinity labeling of bile acid-binding protein. Whatever the reason, specific identification of these newly described cytosolic bile acid-binding proteins (as well as the 20-kDa microsomal binding protein) in the intestine awaits their purification and amino acid sequencing.

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