Immunoprecipitation and MALDI-MS identification of lithocholic acid-tagged proteins in liver of bile duct-ligated rats

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Abstract Formation of covalently bound protein adducts with lithocholic acid (LCA) might explain LCA's known carcinogenic properties and hepatotoxicity. We performed studies aimed at isolating and identifying hepatic proteins tagged with LCA, presumably via the ε-amino group of lysine residues. Antibodies recognizing the 3α-hydroxy-5β-steroid moiety of LCA were generated by immunizing rabbits with immunogens in which the carboxyl group of LCA was coupled to BSA via a 6-aminohexanoic acid and/or succinic acid spacer. The resulting antibodies reacted with *N*-α-(*t*-butoxycarbonyl)-L-lysine-ε-LCA, the amidated and nonamidated forms of LCA, as well as synthetically prepared LCA adducts with ovalbumin and lysozyme. Proteins tagged with LCA in the liver of bile duct-ligated rats were isolated by immunoprecipitation using these antibodies. Proteins were isolated by two-dimensional electrophoresis, and their structure was identified using matrix-assisted laser desorption ionization time-of-flight mass spectrometry and computer-assisted programs. Proteins labeled with LCA were Rab-3, Rab-12, Rab-16, and M-Ras. Rab proteins are Ras-like small GTP binding proteins that regulate vesicle trafficking pathways. The covalent binding of the Rab proteins with LCA may influence vesicular transport or binding of vesicles to their cognate membrane and may contribute to LCA-induced liver toxicity.—Ikegawa, S., T. Yamamoto, H. Ito, S. Ishiwata, T. Sakai, K. Mitamura, and M. Maeda. **Immunoprecipitation and MALDI-MS identification of lithocholic acid-tagged proteins in liver of bile duct-ligated rats. *J. Lipid Res.* 2008.** 49: **2463–2473.**

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Over the past decades, considerable attention has been focused on the possible role of covalent coupling of bile acids to proteins in the pathogenesis of disorders such as cholestasis and colon cancer (1–7). The bile acid that has attracted most interest is lithocholic acid (LCA), a secondary bile acid formed by bacterial 7α-dehydroxylation of chenodeoxycholic acid (CDCA). LCA, a major fecal bile acid in man, is a hydrophobic bile acid that is highly toxic in some experimental animals (8). A protein–bound form of LCA was found in human livers (1, 2), and its concentration was reported to be elevated in the livers of rats treated with a carcinogen, methylazoxymethanol (3). Furthermore, LCA was detected in normal and neoplastic human mammary tissues and neoplasms of the uterus, kidney, lung, and colon (3). It has also been proposed that LCA acts as a promoter of colon cancer (9–17).

One mechanism that has been suggested to explain the carcinogenic properties of LCA is that cellular proteins are covalently modified by the chemically reactive species that are formed during metabolism of LCA (18). LCA metabolism by the hepatocyte is complex. On entering the hepatocyte, LCA is converted to its CoA thioester, which is then transferred to the amino group of glycine or taurine. In addition to this conjugation (or *N*-acyl amidation) process, LCA also may undergo hydroxylation at C-6, C-7, or C-15 (19). In man and mouse, LCA undergoes sulfation at C-3 in addition to *N*-acyl amidation. When incubated with microsomes, LCA may undergo acyl glucuronidation (20–23). Finally adenylation with AMP has also been observed under in vitro conditions (24). In principle, the CoA thioester, the acyl glucuronide, or the adenylate should be capable of binding irreversibly to proteins (18, 25, 26). However, no detailed study on the structural analysis of protein-bound LCA formed in the liver has been reported. Therefore,

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identification of the cellular proteins chemically modified with LCA is essential as a step in the elucidation of the mechanism of LCA-induced cytotoxicity.

Proteomics is the identification of the total proteins from a particular organelle, cell line, tissue, or organism. The most commonly used experimental techniques in proteomics are two-dimensional electrophoresis (2-DE) for separating proteins and mass spectrometry (MS) for the identification of separated proteins. Because of the complexity of the proteome of many biological samples, it is desirable to concentrate the desired analytes from biological samples prior to the MS analysis. Immunoaffinity capture is a powerful protein separation method. This method is based on the specific interaction between an antibody and the target proteins to be captured.

The aim of this work is to show the potential of the immunoaffinity capture of the protein-bound LCA in the liver of the bile duct-ligated rat using a specific antibody with high affinity for the 3α-hydroxy-5β-steroid moiety of LCA. The proteins isolated by this technique could be subjected to structural analysis by the basic proteomic technique of 2-DE, combined with the use of matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) and computer-assisted programs, in order to identify which proteins had formed LCA adducts.

METHODS

Materials

Cholic acid, CDCA, deoxycholic acid, and LCA were purchased from Nacalai Tesque, Inc. (Kyoto, Japan), and ursodeoxycholic acid was kindly donated by Mitsubishi Tanabe Pharma Co. (Osaka, Japan). Glycine-conjugated LCA and taurine-conjugated LCA were prepared by the carbodiimide method (27). *N*-α-(*t*-butoxycarbonyl)-L-lysine-ε-LCA (LCA-N^{α}-BOC-lysine) was synthesized by the activated ester method (28). Lysozyme from chicken egg white (EC 3.2.1.17), BSA, ovalbumin, 3,5-dimethoxy-4-hydroxycinnamic acid [sinapinic acid (SA)], and α-cyano-4-hydroxycinnamic acid (α-CHCA) were purchased from Sigma Chemical Co. (St. Louis, MO). The BCA protein assay kit was from Pierce (Rockford, IL). Immobiline DryStrip gel and imobilized pH gradient (IPG) buffer were obtained from GE Healthcare UK Ltd. Endoproteinase Lys-C (lysylendopeptidase) was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Alkaline phosphatase (ALP) and Zip-Tip C_{18} were purchased from Toyobo (Tokyo, Japan) and Millipore (Billerica, MA), respectively. Freund's complete adjuvant was purchased from Rockland (Gillbertsville, PA). HRP-conjugated anti-rabbit IgG antibody (Fc-specific) and goat anti-rabbit IgG antibody (Fc-specific) were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). HRP-conjugated protein G was purchased from Zymet (East Hanover, NJ). Immobilized protein G beads and normal rabbit IgG protein G beads were obtained from Pierce. Ninety-six-well EIA/RIA plates (No. 3590) were purchased from Corning, Inc. (Corning, MA). EIA/RIA plates coated with goat anti-rabbit IgG antibody were kindly donated by Eiken Chemical Co. Ltd. (Tokyo, Japan). Nitrocellulose membrane (0.45 μm) was obtained from Toyo Roshi Kaisha Ltd. (Tokyo, Japan). All other reagents were of analytical reagent grade and all solvents were purified by distillation prior to use. Water from a Millipore Ultra Water Purification System (Milli-Q Synthesis A10) was used for the preparation of the mobile phase and the aqueous solutions described below.

Buffers

The buffers used for this work were as follows: buffer A, 50 mM NaH_2PO_4-Na_2HPO_4 (pH 7.3); buffer B, buffer A containing gelatin (0.1%, w/v) and NaCl (0.9%, w/v); buffer C, buffer A containing NaCl (0.9%, w/v); buffer D, buffer C containing Tween 20 (0.05%, v/v); buffer E, 1.5 mM KH_2PO_4-8.1 mM Na_2HPO_4 (pH 7.4) containing NaCl (0.8%, w/v) and KCl (0.02%, w/v).

Apparatus

Melting points were measured on an electric microhot stage and are uncorrected. Proton ^{1}H-NMR analysis was performed using a JNM-GS 270 (JEOL, Tokyo) at 270.05 MHz. Chemical shifts are given as the δ value with tetramethylsilane as an internal standard (s, singlet; d, doublet; t, triplet; m, multiplet). The absorbance for ELISA was measured using an MPR A4i microplate reader (TOSOH, Tokyo, Japan). SDS-PAGE and immunoblotting were performed with NA-1013 mini gel slab electrophoresis equipment and an NA-1510B double cassette mini-transfer instrument (Nihon Eido, Tokyo, Japan), respectively. Centrifugal filtration was performed with a 1.5 ml ULTRACENT-10 microconcentrator (TOSOH). The filtrate has a nominal molecular weight cutoff of 10,000, and was used without preconditioning. The chromatograph used in this study was an LC-10 Advp (Shimadzu, Kyoto, Japan) equipped with an ultraviolet (UV) spectrophotometer (215 nm). A CAPCELL PAK C_{18} UG 120 column [5 μm, 150 × 1 mm inner diameter (ID)] (Shiseido Co., Tokyo, Japan) was used at ambient temperature.

MALDI-TOF-MS analysis

Analysis by MALDI-TOF-MS in the positive-ion detection mode was carried out with a Voyager RP (PerSeptive Biosystems, Framingham, MA) equipped with a pulsed nitrogen laser (337 nm). The 5 μl aliquot of the samples in acetonitrile and water (1:1) containing 0.1% (v/v) trifluoroacetic acid was mixed with 5 μl of SA (10 mg/ml) for the protein and/or α-CHCA (10 mg/ml) for the peptide in the same solvent, and 1 μl of the mixture was applied to the stainless steel sample plate and allowed to dry at room temperature and then subjected to MALDI-TOF-MS analysis. The measured *m/z* values were the average mass for the fragment ions. Peptides were initially analyzed in the linear mode with external calibration using the protonated ion $[M+H]^+$ of angiotensin I (1,296.7), ACTH (clip 18–39) (2,465.2), ACTH (clip 7–38) (3,657.9), and insulin (5,733.6). The data were obtained from 256 laser shots using the following parameters: 20.0 kV accelerating voltage, 19.0 kV grid voltage, and 1 kV guide wire voltage.

Synthesis of *N*-(3α-hydroxy-5β-cholan-24-oyl)-6-aminohexanoic acid

To a stirred solution of LCA (1 g) in dioxane (15 ml) were added *p*-nitrophenol (0.74 g) and 1-ethyl-3-(3-dimethylaminopropyl) carbonyldiimide hydrochloride (1.1 g) at room temperature, and the mixture was stirred for 24 h at room temperature. The resulting mixture was diluted with ethyl acetate, washed with H_2O, and dried over anhydrous Na_2SO_4. After evaporation of the solvent in vacuo, the crude product was purified by column chromatography on a silica gel using toluene-acetone (10:1; v/v) as an eluent, to give LCA *p*-nitrophenyl ester (1.2 g). To the ester (300 mg) dissolved in pyridine (6 ml) was added a solution of 6-aminohexanoic acid (158 mg) in 1% NaOH (1 ml). The mixture, after having been kept stirred overnight at room temperature, was acidified

to pH 2.0 with 5% HCl. The resulting precipitates were collected by filtration and then purified by column chromatography on a silica gel using chloroform-methanol (10:1; v/v) as an eluent. Recrystallization of the eluate from methylene chloride-methanol gave the 6-aminohexanoic acid derivative (261 mg) as colorless crystalline product. Melting point 86–92°C. Anal. Calcd for $C_{30}H_{51}O_4N \cdot 1/4H_2O$: C, 72.90; H, 10.50; N, 2.83. Found: C, 72.53; H, 10.25; N, 2.79. ^{1}H-NMR ($CDCl_3$) δ:0.65 (3H, s, 18-H), 0.92 (3H, s, 19-H), 0.92 (3H, d, J = 6.1 Hz, 21-H), 2.37 (2H, t, J = 8.1 Hz, -NHCH$_2$CH$_2$CH$_2$CH$_2$C$\underline{H_2}$CO-), 3.25 (2H, m, -NHC$\underline{H_2}$CH$_2$-), 3.63 (1H, m, 3β-H), 5.67 (1H, t, J = 7.6 Hz, -CON$\underline{H}$CH$_2$-).

Synthesis of *N*-(3α-hydroxy-5β-cholan-24-oyl)-6-aminohexanoic acid succinimidyl ester

To a stirred solution of the 6-aminohexanoic acid derivative (261 mg) in 95% dioxane (13 ml) were added 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (204 mg) and *N*- hydroxysuccinimide (123 mg). The mixture, after having been kept stirred overnight at room temperature, was diluted with ethyl acetate, washed with H_2O, and dried over anhydrous Na_2SO_4. After evaporation of the solvent in vacuo, the crude product was purified by column chromatography on a silica gel using chloroform-methanol (10:1; v/v) as an eluent to give the succinimidyl ester (95 mg) as colorless crystalline product. mp 83.5–88°C. Anal. Calcd for $C_{34}H_{54}O_5N_2 \cdot H_2O$: C, 69.35; H, 9.59; N, 4.91. Found: C, 68.69; H, 9.14; N, 4.88. ^{1}H-NMR ($CDCl_3$) δ:0.64 (3H, s, 18-H), 0.92 (3H, s, 19-H), 0.92 (3H, d, J = 6.0 Hz, 21-H), 2.63 (2H, t, J = 7.0 Hz, -NHCH$_2$CH$_2$CH$_2$CH$_2$C$\underline{H_2}$CO-), 3.26 (2H, m, -NHC$\underline{H_2}$CH$_2$-), 3.61(1H, m, 3β-H), 5.66 (1H, t, J = 5.5 Hz, -CONH$\underline{C}$H$_2$-).

Synthesis of 3α-formyloxy-24-hydroxy-5β-cholane

To a stirred solution of LCA (2.0 g) in formic acid (20 ml) was added dropwise perchloric acid (60%) (0.4 ml) at 0°C, whereupon it was allowed to stir at the same temperature for 20 min. After addition of acetic anhydride (2 ml) under ice cooling, the resulting mixture was poured into ice water. The resulting precipitates were collected by filtration and dried in vacuo under P_2O_5. To the crude product (1.99 g) dissolved in anhydrous tetrahydrofurane (10 ml) were added dropwise triethylamine (1.36 ml) and ethyl chlorocarbonate (0.94 ml) under ice cooling, whereupon it was allowed to stir at the same temperature for 30 min, after which $NaBH_4$ (0.6 g) in H_2O (0.2 ml) was added dropwise to it. Stirring was continued for 1 h, after which acetic acid was added dropwise to decompose the excess reagent and extracted with ethyl acetate. The extract was washed with H_2O, 5% $NaHCO_3$, and saturated brine, and then dried over anhydrous Na_2SO_4. After evaporation of the solvent in vacuo, the crude product was purified by column chromatography on a silica gel using toluene-acetone (20:1; v/v) as an eluent to give the 3α-formyloxy-24-alcohol (1.09 g). ^{1}H-NMR ($CDCl_3$) δ: 0.65 (3H, s, 18-H), 0.91 (3H, s, 19-H), 0.92 (3H, d, J = 6.0 Hz, 21-H), 3.61 (2H, t, J = 7.0 Hz, 24-H), 4.85 (1H, m, 3β-H), 8.00 (1H, s, -OCOH).

Synthesis of 3α-formyloxy-24-hemisuccinoyloxy-5β-cholane

A mixed solution of 3α-formyloxy-24-alcohol (765 mg) and succinic anhydride (392 mg) in pyridine (4 ml) was heated at 90°C for 9 h, after which the reaction mixture was diluted with ethyl acetate, washed with H_2O, and dried over anhydrous Na_2SO_4. After evaporation of the solvent in vacuo, the crude product was purified by column chromatography on a silica gel using *n*-hexane-ethyl acetate (7:1; v/v) as an eluent. Recrystallization of the eluate from acetone-hexane gave the 3α-formyloxy-24-hemisuccinate (1.04 g) as colorless plate. mp 111–114°C. ^{1}H-NMR ($CDCl_3$) δ: 0.65 (3H, s, 18-H), 0.95 (3H, s, 19-H), 0.93 (3H, d, J = 6.0 Hz, 21-H), 2.62 (4H, m, -CO(C$\underline{H_2}$)$_2$COOH), 4.06 (2H, m, 24-H), 4.85 (1H, m, 3β-H), 8.05 (1H, s, -OCOH).

Synthesis of 3α-hydroxy-24-hemisuccinoyloxy-5β-cholane

To a solution of 3α-formyloxy-24-hemisuccinate (1.04 g) in methanol (20 ml) was added 5% $NaHCO_3$ (5 ml), after which the mixture was stirred at 60°C for 2 h. After evaporation of the solvent in vacuo, the residue was diluted with ethyl acetate, washed with H_2O, and dried over anhydrous Na_2SO_4. The solvent was evaporated to dryness in vacuo to give the 3α-hydroxy-24-hemisuccinate (157 mg) as colorless crystalline. mp 111–114°C. Anal. Calcd for $C_{28}H_{46}O_5$: C, 72.69; H, 10.02. Found: C, 72.44; H, 10.06. ^{1}H-NMR ($CDCl_3$) δ: 0.64 (3H, s, 18-H), 0.92 (3H, s, 19-H), 0.91 (3H, d, J = 6.0 Hz, 21-H), 2.65 (4H, m, -CO(C$\underline{H_2}$)$_2$COOH), 3.64 (1H, m, 3β-H), 4.06 (2H, m, 24-H).

Synthesis of 3α-hydroxy-24-hemisuccinyloxy-5β-cholane succinimidyl ester

To a solution of 3α-hydroxy-24-hemisuccinate (300 mg) in dioxane (5 ml) were added 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (250 mg), *N*-hydroxysuccinimide (150 mg) and H_2O (0.5 ml). The mixture, after having been kept stirred overnight at room temperature, was diluted with ethyl acetate, washed with H_2O, and dried over anhydrous Na_2SO_4. After evaporation of the solvent in vacuo, the crude product was purified by column chromatography on a silica gel using toluene-acetone (3:1; v/v) as an eluent to give the 24-succinimidyl ester (150 mg) as colorless crystalline product. mp 145–154°C. Anal. Calcd for $C_{32}H_{50}NO_7 \cdot 3/2H_2O$: C, 65.39; H, 9.08; N, 2.38. Found: C, 65.02; H, 8.96; N, 2.37. ^{1}H-NMR ($CDCl_3$) δ: 0.64 (3H, s, 18-H), 0.91 (3H, d, J = 6.0 Hz, 21-H), 0.92 (3H, s, 19-H), 2.74 (2H, t, J = 7.0 Hz, -COCH$_2$C$\underline{H_2}$COOH), 2.96 (2H, t, J = 7.0 Hz, -COC$\underline{H_2}$CH$_2$COOH), 3.61 (1H, m, 3β-H), 4.09 (2H, m, 24-H).

Synthesis of LCA acyl-adenylate

LCA acyl-adenylate (LCA-AMP) was chemically synthesized by the method previously reported (26). The synthetic products were purified by column chromatography on Sephadex LH-20 (40 × 1.5 cm ID) using 40% methanol as the eluent at a flow rate of 18 ml/h. Fractions (each 0.3 ml) having a spot with a rate of flow value of 0.55 for LCA-AMP on TLC were collected, and the solvent was removed in vacuo, to give the desired compound (4% yield) as colorless amorphous substances that were kept at −80°C. The structures of the purified LCA-AMP were confirmed by ^{1}H-NMR (CD_3OD) δ: 0.50 (3H, s, 18-H), 0.87 (3H, s, 19-H), 0.95 (3H, d, J = 5.4 Hz, 21-H) 3.65 (3H, m, 3β-H), 4.16 and 4.30 (each 1H, m, 4′- and 5′-H), 4.44 and 4.63 (each 1H, t, J = 5.4 Hz, 2′- and 3′-H), 6.11 (1H, d, J = 5.4 Hz, 1′-H), 8.28 and 8.59 (each 1H, s, 2″- and 8″-H), and the negative-ion ESI mass spectrum: $[M-H]^-$ at *m/z* 704.

Preparation of immunogen and enzyme-labeled antigen

Two kinds of hapten-carrier conjugates (**10** and **11**) were prepared by the reaction of the succinimidyl esters (**4** and **8**) with BSA (~60 mol equiv. to BSA). The hapten/BSA molar ratio was determined to be 19 for the **10** and 28 for the **11** by titration of the residual free amino groups on BSA using trinitrobenzensulfonic acid. To obtain enzyme-labeled antigen, **4** was reacted with 0.2 mg of ALP in 0.1 M carbonate buffer (pH 9.0)-dioxane (1:1; v/v, 400 μl) at 4°C for 4 h. The molar ratio of the activated ester to enzyme in this reaction was adjusted to 40. Removal of unreacted steroid by dialysis afforded the desirable enzyme-labeled LCA, which was stored at 4°C until use.

Immunization of rabbit for production of anti-LCA antiserum

The hapten-BSA conjugate (1.5 mg) was dissolved in sterile saline (0.5 ml) and emulsified with Freund's complete adjuvant (0.5 ml). The emulsion was injected into domestic female albino rabbits (1.5–2 kg body weight) subcutaneously at multiple sites over the back. This procedure was repeated at 1 week intervals during the first month and then once every month. The antisera prepared from blood by centrifugation at 3,000 rpm for 10 min was stored at 4°C with NaN_3 (0.1%, w/v).

Characterization of antibodies

A solution of antiserum suitably diluted with buffer B (100 μl) was distributed in each well of a goat anti-rabbit IgG antibody-coated microplate. After incubation at 37°C for 1 h, the solutions were aspirated off and the wells were washed three times with buffer D. The ALP-labeled LCA (200 ng) in buffer B (100 μl) and standard bile acid solutions (50 μl) diluted with 50% methanol were then added, mixed, and incubated at 37°C for 2 h. After washing in the same manner, bound enzyme activity on the plate was colorimetrically measured using a substrate solution (100 μl) containing 1 mM *p*-nitrophenyl phosphate disodium salt and $MgCl_2$ (0.01%, w/v) in 50 mM carbonate buffer (pH 10). After incubation at 37°C for 60 min, the enzymatic reaction was terminated by the addition of 0.1 M NaOH (100 μl). The absorbance at 415 nm was measured using an MPR A4i microplate reader.

Immunological detection of LCA ovalbumin adducts

A solution of ovalbumin (2 mg) diluted with buffer A (100 μl) was distributed in each well of the 96-well EIA/RIA plates, which were left overnight at room temperature. After washing three times with buffer C, LCA-AMP (0, 1, and 10 μg) in 50% methanol (100 μl) was added to the wells. After incubation at 37°C for 24 h, the solutions were aspirated off and the wells were washed three times with buffer C. The wells were blocked with 5% skim milk solution in buffer C (300 μl) at 37°C for 2 h, and washed three times with buffer C. A 1:10,000 (v/v)-diluted antiserum (100 μl) diluted with buffer B was added to the wells, which were incubated at 37°C for 1 h. After washing three times with buffer B, a solution (100 μl) of 1:20,000-diluted HRP-labeled anti-rabbit IgG antibody diluted with buffer B was added and incubated at 37°C for 1 h. After washing as already described, a citrate-phosphate buffer (pH 5.0) containing 0.04% *o*-phenylenediamine·2HCl and 0.018% H_2O_2 was distributed to the wells, and the plates were incubated at room temperature for 15 min. The enzymatic reaction was terminated by the addition of 1 M H_2SO_4 (100 μl), and the absorbance at 492 nm was measured using an MPR A4i microplate reader.

Preparation of lysozyme-LCA adduct and structural elucidation

LCA-AMP (500 μg) was incubated with lysozyme (1 mg) in buffer C (1 ml) at 37°C for 96 h, and the reaction was stopped by addition of acetic acid (50 μl). The mixture was subjected to centrifugal filtration to remove any low-molecular-weight substances, followed by centrifugation in buffer A. A portion of the mixture was then subjected to reductive *S*-alkylation and proteolytic digestion with endoproteinase Lys-C, as follows. The mixture (418 μg) was reduced with DTT (8.64 μg) in 310 μl of 0.1 M Tris-HCl buffer (pH 8.1) containing 8 M urea and 2 mM EDTA for 15 min at 50°C, and carboxymethylated with sodium iodoacetate (11.6 μg) at room temperature in the dark for 1 h under N_2 atmosphere. A solution of endoproteinase Lys-C (0.4 μg) in H_2O (10 μl) was added to the reaction mixture, and the resulting mixture was incubated at 37°C for 4 h. The reaction was terminated by addition of acetic acid (100 μl). After desalting the mixture with Zip-Tip C_{18}, 1 μl of the mixture was subjected to MALDI-TOF-MS analysis.

Immunoblotting analysis of lysozyme-bound LCA

For SDS-PAGE, 10 μg protein was diluted to 1:1 with 125 mM Tris-HCl buffer (pH 6.8) containing 4% SDS, 2% 2-mercaptoethanol, 20% glycerol, and 0.02% pyronin Y, heated at 100°C for 5 min, and resolved on 15% acrylamide gels. Proteins resolved by polyacrylamide gels were transblotted to 0.45 μm nitrocellulose membrane at 55 V for 1 h in 25 mM Tris-192 mM glycine buffer (pH 8.3). After transferring, the blots were blocked by shaking overnight at 4°C in buffer C containing 5% skim milk. The blotted membranes were incubated with 1:1,000-diluted anti-LCA antiserum (Ab_2) for 3 h at room temperature with shaking. The blots were rinsed for three 5 min washes in buffer D and two 5 min washes in buffer C. The immunoblots were incubated for 1 h in 1:1,000-diluted goat anti-rabbit IgG diluted with buffer C. After washing as above, the immunoblots were incubated for 1 h with 1:1,000-diluted HRP-labeled protein G. The nitrocellulose membranes were again washed extensively as above, and the immunoreactive proteins were visualized with 0.05% (w/v) 3,3′-diaminobenzidine containing 0.03% H_2O_2 in buffer C for 5 min.

Immunoprecipitation of protein-bound LCA in the liver from bile duct-ligated rat

Male Wistar rats (230–250 g body weight) were obtained from Japan SLC. Inc. (Shizuoka, Japan), and used with approval from the Kinki University Committee for the Care and Use of Laboratory Animals, which conforms to National Institutes of Health guidelines. The rats were anesthetized with diethyl ether, and the common bile duct was ligated. Rats had free access to food and water. Animals were fasted overnight and then euthanized by decapitation under ether anesthesia. The liver (10 g wet weight) was minced and homogenized in 3 vol ice-cold 10 mM Tris-HCl buffer (pH 7.3) containing 0.25 M sucrose and 1 mM KCl by 10 rapid strokes using a motor-driven Teflon-glass homogenizer. The homogenate was centrifuged at 600 *g* for 10 min, followed by further centrifugation at 8,000 *g* for 10 min. The resulting supernatant was centrifuged at 105,000 *g* for 1 h. The supernatant was dialyzed against cold running water at 4°C for 48 h to remove low-molecular-weight substances, and was kept at −70°C until analysis.

One milliliter of supernatant (1 mg protein) was incubated with immobilized protein G beads (10 μl) and normal rabbit IgG-immobilized protein G beads (10 μl) at 4°C for 1 h, and then centrifuged at 15,000 rpm for 1 min. The supernatant was divided into two portions, and 1:1,000-diluted anti-LCA antiserum (Ab_2) (10 μl) and normal rabbit serum (10 μl) were added to each portion, and the resulting mixtures were incubated at 4°C for 3 h. After addition of protein G beads (10 μl), the mixture was incubated at 4°C for 1 h and then centrifuged at 2,000 rpm for 1 min. All the liquids were removed using a flat pipette tip and replaced with 400 μl of buffer C. The immunoprecipitates were washed three times with buffer C, and then subjected to 2-DE analysis.

2-DE of proteins and in-gel digestion

The immunoprecipitate (10 μl) was diluted with lysis buffer [7 M urea, 2 M thiourea, 5% CHAPS, 2% IPG buffer (pH 4–7) and 50 mM DTT] (70 μl), and allowed to stand at room temperature for 30 min. After centrifugation at 20,000 *g* for 5 min, the supernatant was diluted in rehydration buffer [8 M urea, 0.5%

CHAPS, 0.5% IPG buffer, 20 mM DTT, and 0.04% bromophenol blue (BPB)] (70 μl), and was shaken at room temperature for 5 min. After centrifugation at 20,000 *g* for 5 min, the supernatant was loaded onto Immobiline DryStrip gels (pH 4–7, 7 cm) and rehydrated overnight. Isoelectric focusing was performed at 30°C using a multi-step protocol (200 V, 30 min; 400 V, 30 min; 1,000 V, 1 h; 2,000 V, 18 h). At the end of focusing, individual strips were equilibrated for 40 min with equilibration buffer [6 M urea, 30% glycerol, 1% SDS, 17.7 mM DTT, 0.04% BPB, 50 mM Tris-HCl buffer (pH 8.7)] (5 ml). For the second dimension, the proteins were separated by SDS-PAGE, which was performed at a constant voltage of 150 V using a 15% polyacrylamide separation gel for 80 min. The gel was stained with Silver Stain MS Kit (Wako Pure Chemical Industries, Japan). Protein spots were excised from the gel and placed in 1.5 ml microtubes and destained in 100 μl of destain solution containing 15 mM $K_3[Fe(CN)_6]$ and 50 mM $Na_2S_2O_3$. The gel slices were washed for 30 min in 100 μl of 50% acetonitrile in 25 mM ammonium bicarbonate (pH 8.6) and then dried under a vacuum. Disulfide bonds were reduced with 10 mM DTT in the same buffer (100 μl) by incubation for 1 h at 56°C and alkylated with 55 mM iodoacetamide in the same buffer (100 μl) for 1 h in the dark at room temperature. The gel slices were washed twice. After the buffer was discarded, the gel pieces were dehydrated with 50% acetonitrile in 25 mM ammonium bicarbonate (pH 8.6) and then dried by vacuum centrifugation. The gel pieces were then reconstituted in 50 μl of digestion buffer containing 50 ng of sequencing-grade modified trypsin (Promega, Madison, WI) at 0°C for 30 min. Excess reagents were removed, and the gel slices were incubated at 37°C for 18 h. Then the gel slices were suspended in 10 μl extract buffer (3% acetonitrile, 2% trifluoroacetic acid in water) and the supernatant was desalted in Zip-Tip C_{18} micro-columns and submitted to MALDI-TOF-MS analysis.

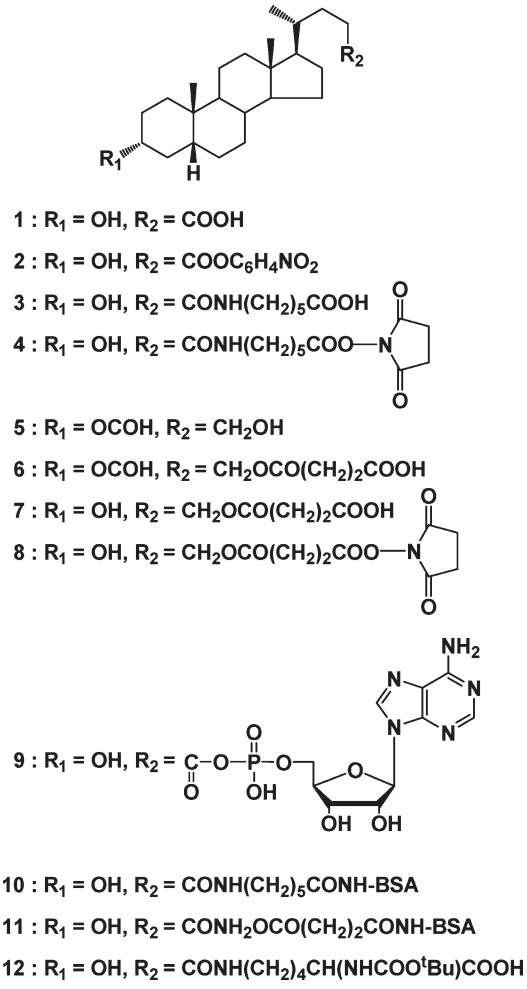

Fig. 1. Structures of lithocholic acid (LCA), its related compounds, and the immunogen.

TABLE 1. Binding abilities of anti-LCA antibodies as determined by competitive ELISA

Compound	Cross-reactivity		
	Ab_1	Ab_2	Ab_3
		%[a]	
LCA-N^{α}-BOC-Lysine	100	100	100
LCA	920	44	100
GLCA	468	14	20
TLCA	757	56	127
LCA-6AH[b]	1,923	——-	——-
LCA-HS[c]	——-	3,090	1,735
UDCA	0.63	0.19	0.71
CDCA	1.07	0.24	1.07
DCA	0.40	0.19	0.32
CA	0.40	0.02	0.05
Titer	1:500	1:160,000	1:90,000
Midpoint	150 ng	6.8 ng	9.2 ng

LCA, lithocholic acid; GLCA, glycine-conjugated LCA, TLCA, taurine-conjugated LCA; UDCA, ursodeoxycholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; CA, cholic acid.
[a] Calculated by the 50% displacement method.
[b] *N*-(3α-hydroxy-5β-cholan-24-oyl)-6-aminohexanoic acid.
[c] 3α-hydroxy-24-hemisuccinoyloxy-5β-cholane.

RESULTS AND DISCUSSION

Preparation of anti-LCA antibody

To capture protein-bound LCA by immunoprecipitation, the antibody should have the ability to bind to the steroidal moiety of protein-bound LCA, where LCA is coupled via the ε-amino group of lysine residue on the proteins. Several studies have shown that the binding of

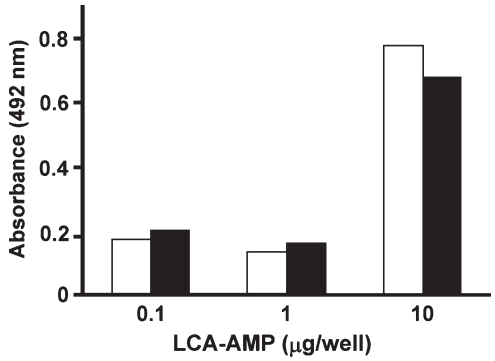

Fig. 2. Immunological detection of LCA residue on ovalbumin molecule introduced by the reaction of LCA acyl-adenylate (LCA-AMP) with lysozyme. Open and closed columns represent the results of Ab_2 and Ab_3, respectively. Numbers at the bottom indicate the amount (μg/well) of LCA-AMP.

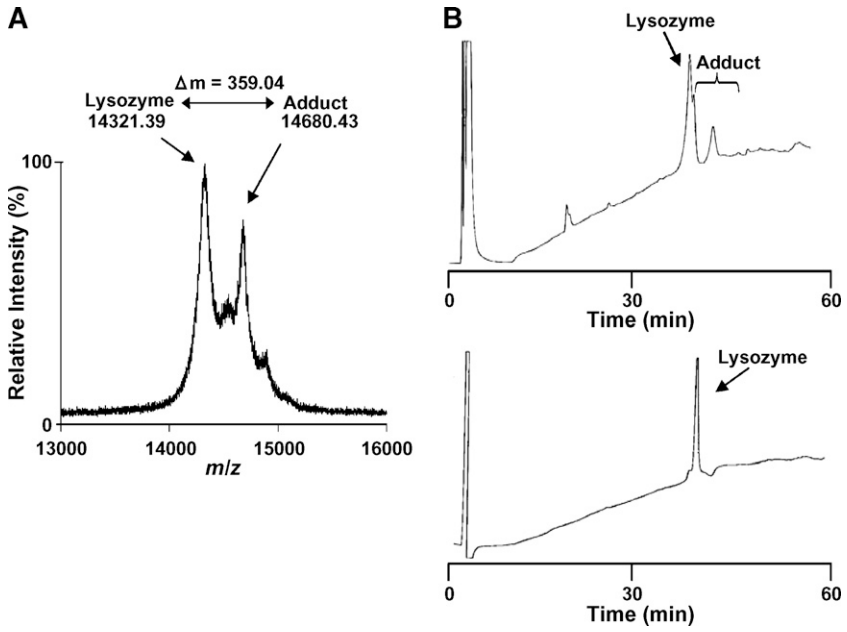

Fig. 3. Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrum (A) and high-performance liquid chromatogram (B) of an incubation mixture of LCA-AMP with lysozyme. HPLC conditions: column, CAPCELL PAK C_{18} UG 120 (5 μm, 150 mm × 1.0 mm inner diameter); mobile phase, solvent A (0.1% trifluoroacetic acid in H_2O) and solvent B (0.1% trifluoroacetic acid in CH_3CN) from 0:100 to 70:30 over 70 min; flow rate, 50 μl/min; detection, 215 nm.

antibodies is improved when the hapten is separated spatially from the coupling site with a carrier protein such as BSA and keyhole limpet hemocyanin. If the chemical group involved in the linkage between the hapten and the carrier is bound to the recognition site, it is desirable to increase the distance between the steroidal moiety and the carrier protein to increase the probability of raising antibodies with high recognition (29–32). Accordingly, we designed immunogens in which the terminal carbon of the side chain of LCA was coupled to BSA via a 6-aminohexanoic acid and/or a succinic acid spacer to produce antibodies specific for the steroid moiety (**Fig. 1**). Initial efforts were, therefore, directed to preparing the haptenic derivatives of LCA coupled with 6-aminohexanoic acid and succinic acid as haptenic derivatives. The former compound (LCA-6AH) was prepared by the activated ester method via LCA *p*-nitrophenyl ester. On the other hand, the latter compound (**7**) was prepared from LCA in four steps. Thus, the 3α-hydroxyl group of LCA was protected as a formyl ester, and the carboxyl group was reduced to a primary alcohol by reducing the mixed anhydride with $NaBH_4$. The hemisuccinate (**6**) was then formed by esterification with succinic anhydride in pyridine. Upon treatment with $NaHCO_3$, the formyl group was readily removed to produce the desired hapten (LCA-HS, **7**). These haptenic derivatives were then converted to their corresponding succinimidyl esters (**4** and **8**), which were coupled to BSA by an activated ester method to produce LCA-BSA conjugates. A satisfactory number of hapten molecules (19 for **10** and 28 for **11**) were incorporated in each conjugate. These immunogens were then sub-

TABLE 2. Calculated and observed peptide fragments covalent adducts formed from LCA-AMP and lysozyme

Peak Number	Amino Acid Residue		Sequence	$[M+H]^+$		Binding Site
	From	To		Calculated	Observed	
				m/z		
1	2	13	VFGRC[b]ELAAAMK	1,353.7	1,353.7	
2	117	129	GTDVQAWIRGC[b]RL	1,532.8	1,532.5	
3	1	13	K[a]VFGRCELAAAMK[a]	2,140.9	2,140.5	K-1 and K-13
4	98	116	IVSDGNGMNAWVAWRNRC[b]K	2,235.1	2,236.8	
5	14	33	RHGLDNYRGYSLGNWVCAAK	2,338.1	2,339.9	
6	97	116	K[a]IVSDGNGMNAWVAWRNRC[b]K or KIVSDGNGMNAWVAWRNRC[b]K[a]	2,721.7	2,722.4	K-97 or K-166
7	34	96	FESNFNTQATNRNTDGSTDYGILQINSRWWC[b] NDGRTPGSRNLC[b]NIPC[b]SALLSSDITASVNC[b]AK	7,129.2	7,132.0	

[a] Presumed binding site of LCA.
[b] Carboxymethylated.

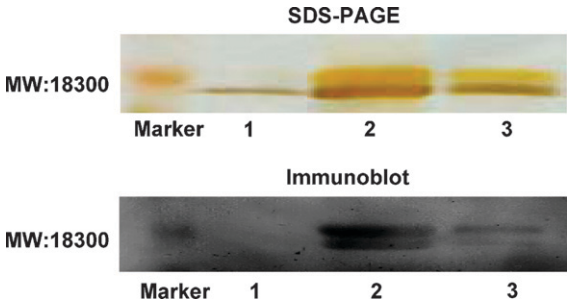

Fig. 4. SDS-PAGE and immunoblot analysis of incubation mixture of LCA-AMP and lysozyme. Immunoblotting (1 and 2 μg/lane) was carried out as described in the Methods section.

cutaneously injected into individual rabbits with Fruend's complete adjuvant. The titer of the antibody was checked at each stage of the immunization regimen by a competitive ELISA system using an enzyme-labeled hapten, and ALP-labeled LCA 6-aminohexanoic acid conjugate as a probe. The antisera obtained at 3 and 6 months after the initial immunization were shown by ELISA to have optimal dilutions of 500 for Ab_1, 160,000 for Ab_2, and 90,000 for Ab_3, and feasible dose-response curves for LCA-N^{α}-BOC-lysine in the range of 20 ng–5 μg per assay. An inhibition test was further performed by the addition of bile acid derivatives to compete with an enzyme-labeled antigen for binding to the antibody, and the cross-reactivities were determined by the 50% displacement method, taking the reactivity with LCA-N^{α}-BOC-lysine as 100%. Results are shown in **Table 1**. As expected, the antibody (Ab_1) obtained from LCA-6-aminohexanoic acid-derivative BSA conjugate showed high reactivity for amidated and nonamidated forms of LCA and haptenic derivatives, whereas the reactivities toward other common bile acids in human body fluids were significantly lower. Similar phenomena were observed for the antibodies (Ab_2 and Ab_3) obtained by using LCA succinic acid-derivative BSA conjugate as the immunogen. However, the magnitudes of cross-reactivities to the above-mentioned compounds was somewhat low (14–127%) except for the high reactivity (3,090% and 1,735%) with the haptenic derivatives. These results are ascribable to the site of conjugation, because LCA was coupled with BSA through the bridge at the C-24 position, remote from the steroidal moiety. Hence, in immunoblotting, the developed antisera may be useful for detection of LCA anchored on proteins.

Immunological detection of LCA residues anchored on proteins

The antibody was evaluated for its utility in the detection of LCA residues by using ovalbumin-bound LCA as a model modified protein. In the experiment, fixed amounts of LCA placed on the microtiter plate (2 mg of ovalbumin per well) were incubated with various amounts of LCA-AMP, a reactive metabolite of LCA, at 37°C for 2 days. After removal of unreacted materials with washings, the LCA residues anchored on the formed adducts were determined by a sandwich-type ELISA using 1:10,000-diluted antibodies (Ab_2 and Ab_3) and HRP-labeled anti-rabbit IgG antibody. As shown in **Fig. 2**, increased signal intensity with 10 μg of LCA-AMP placed on the microwell was observed, demonstrating the binding against LCA anchored on ovalbumin. Thus, this antibody appears to be useful for selective detection of LCA-protein adducts by immunoblotting.

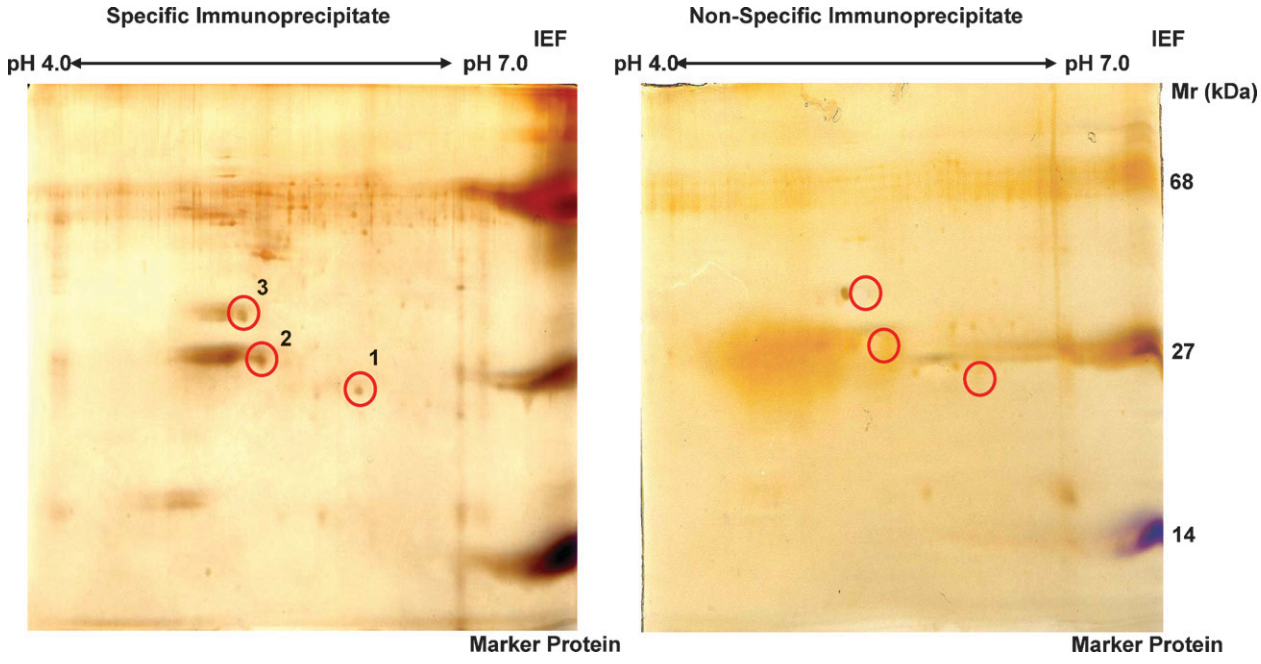

Fig. 5. Two-dimensional (2-DE) pattern of (left) specific immunoprecipitates and (right) nonspecific immunoprecipitates. Each 10 μg of protein of the samples was subjected to 2-DE system (first dimension, immobilized pH linear gradient, pH 3–10; second dimension, 10–20% SDS-PAGE). Proteins were visualized by silver staining. Selected proteins identified by excision and in-gel digestion followed by MS are indicated with spot numbers.

In an effort to develop immunoabsorption for selective detection of protein-bound LCA by using antiserum, lysozyme-bound LCA as a model modified protein was prepared by incubation of lysozyme with 10-fold molar of LCA-AMP in phosphate buffer (pH 7.4) at 37°C for 96 h, and analysis of the reaction mixtures was carried out by MALDI-TOF-MS in the linear mode. The mass spectrum showed an abundant ion at 14,680.43, along with the $[M+H]^+$ of unmodified lysozyme (**Fig. 3A**). The *m/z* value was shifted by 359.04 Da from the *m/z* 14,321.39 of unmodified lysozyme, indicating that the lysozyme had been modified by the addition of one molecule of LCA. Subsequent liquid chromatographic separation of the incubated reaction mixture was carried out on a CAPCELL Pak C_{18} column in gradient elution mode, monitored by UV detection at 215 nm (Fig. 3B). The unmodified and modified lysozyme samples were effectively resolved, showing the presence of at least two kinds of lysozyme-bound LCAs in the incubation mixture. Further analysis of the protein modification using whole mixture was carried out by MALDI-TOF-MS of peptide fragment mixtures obtained by proteolytic digestion with endopeptidase Lys-C after reduction with dithiothreitol and alkylation with iodoacetic acid of the reaction mixture. The mass spectrum showed several protonated ions $[M+H]^+$ of the peptide fragments. As listed in **Table 2**, the ions at 1,353.7, 1,532.5, 2,236.8, 2,339.9, and 7,132.0 were the Lys-C peptides corresponding to residues 2–13, 117–129, 98–116, 14–33, and 34–96. The ion at *m/z* 2,140.5 was shifted by two molecules of LCA from the *m/z* 1,481.9 of the peptide corresponding to residues 1–13, indicating that the site of covalent binding was the K residue. In addition, an ion at *m/z* 2,722.4 also indicated the existence of peptide residues 97–116 with one molecule of LCA. It is known that lysozyme has six lysine residues: 1, 13, 33, 96, 97, and 116. It has also been reported that the endoproteinase Lys-C cleaves an amide bond at the terminus side of lysine, and that its activity is inhibited by acylation of the ε-amino group of lysine (33). Hence, it seems likely that the binding sites of LCA were the Lys-1 and -13, and -97 or -166 residues, respectively, in the peptide bonds of peaks number 3 and 6. As a result, the covalent LCA-lysozyme adducts bound through Lys-1, Lys-13, Lys-97, and Lys-116 of the protein were definitely confirmed.

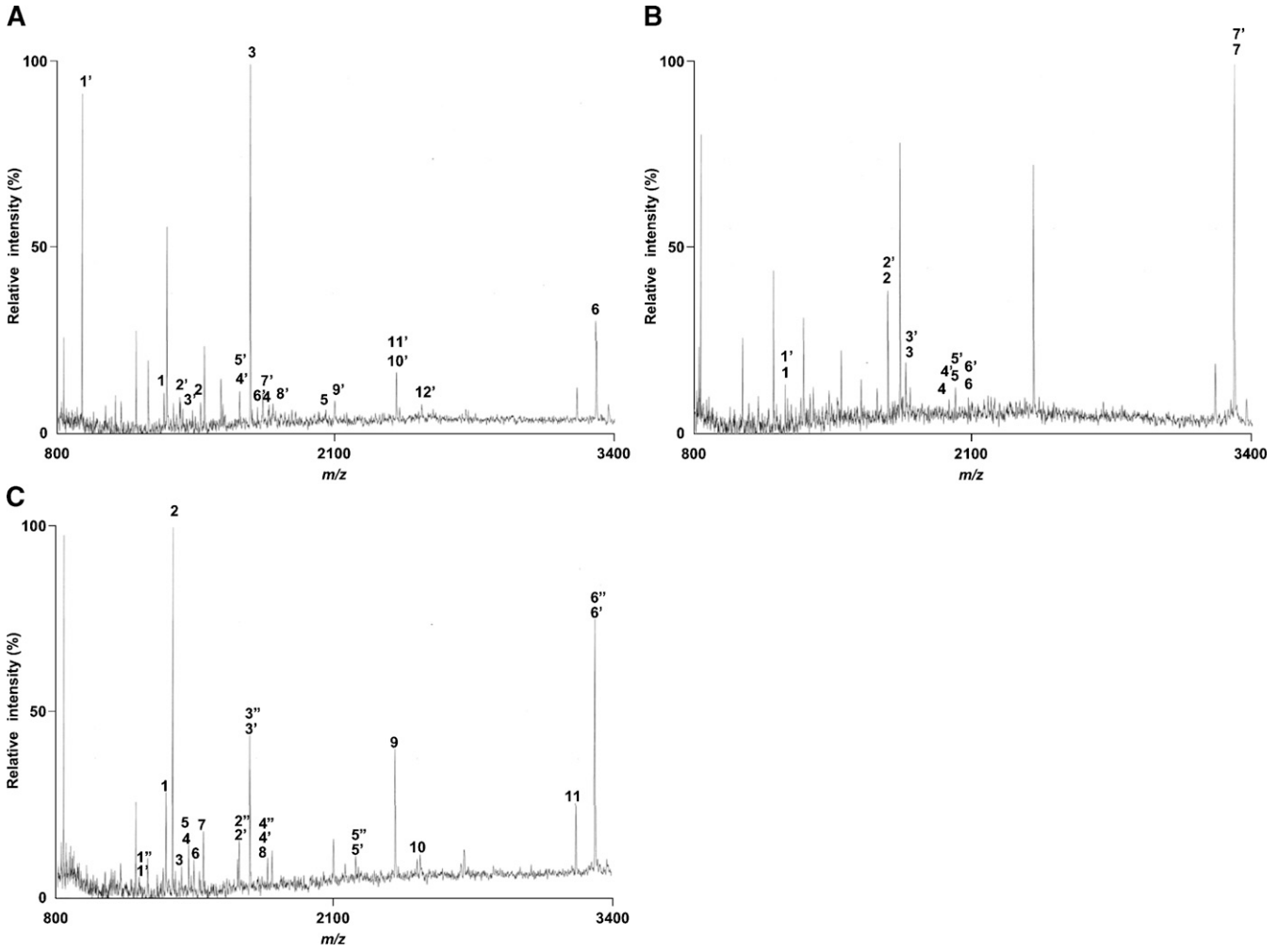

Fig. 6. MALDI-TOF mass spectra of peptides obtained from in-gel digestion of (A) protein spot 1, (B) protein spot 2, and (C) protein spot 3 excised from the gel shown in Fig. 5.

TABLE 3. Calculated and observed ions of trypsin digests of reduced and alkylated proteins in spots 1

Protein Name	Peak Number	Amino Acid Sequence From	To	Sequence	$[M+H]^+$ Calculated	Observed
					m/z	
Rab-3	1	84	93	YRTITTAYYR	1,307.7	1,308.1
(33.5)[d]	2	13	24	DAADQNFDYMFK	1,464.6	1,463.9
	3	122	136	TYSWDNAQVILVGNK	1,707.9	1,707.8
	4	63	70	TVYR[a]HDK[a]R or VYR[a]HDKR[a] or TVYRHDK[a]R[a]	1,791.2	1,792.2
	5	84	93	YR[a]TITTAYYR[a]	2,024.2	2,022.9
	6	187	219	M[b]NESLEPSSSPGSNGKGPALGDTPPPQPSSC[c]GC[c]	3,315.4	3,314.1
M-Ras	1′	106	112	FHQLILR	926.6	972.4
(43.2)[d]	2′	136	147	VTRDQGKEMATK	1,379.7	1,379.8
	3′	128	138	VDLM[b]HLRKVTR	1,383.9	1,383.7
	4′	148	158	YNIPYIETSAK[a]	1,656.9	1,658.5
	5′	194	202	GDR[a]ATGTHK[a]	1,659.1	1,658.5
	6′	128	138	VDLM[b]HLR[a]KVTR or VDLM[b]HLRK[a]VTR or VDLM[b]HLRKVTR[a]	1,742.1	1,741.7
	7′	159	173	DPPLNVDKTFHDLVR	1,765.9	1,766.5
	8′	174	182	VIR[a]QQVPEK[a]	1,813.2	1,813.6
	9′	136	147	VTR[a]DQGK[a]EM[b]ATK or VTR[a]DQGKEM[b]ATK[a] or VTRDQGK[a]EM[b]ATK[a]	2,096.3	2,097.9
	10′	115	134	DRESFPMILVANKVDLMHLR	2,384.3	2,385.1
	11′	194	208	GDR[a]ATGTHK[a]LQC[c]VIL	2,385.5	2,385.1
	12′	117	134	ESFPM[b]ILVANK[a]VDLM[b]HLR or ESFPM[b]ILVANKVDLM[b]HLR[a]	2,503.4	2,502.1

[a] Presumed binding site of LCA.
[b] Oxidated.
[c] Carbamoylmethylated.
[d] Percent of covered amino acids.

Accordingly, the above-mentioned samples were separated by SDS-PAGE, followed by blotting to a nitrocellulose membrane. The blotted membranes were incubated with 1:1,000-diluted Ab_2, and consecutively incubated with goat anti-rabbit IgG and HRP-labeled protein G, followed by staining with 3,3′-diaminobenzidine and H_2O_2. As shown in **Fig. 4**, the immunoblot results exhibited a strongly stained band in lanes 2 and 3 loaded with LCA-lysozyme adducts, although no significantly stained bands in lane 1 loaded with lysozyme were observed. These results indicate that antibody bound to the LCA-lysozyme adducts and that such binding could be attributed to immunorecognition of the LCA bound to the lysine residues of lysozyme.

The experiments cited above indicate that our polyclonal antibodies were able to detect protein-bound LCA. The antibodies were able to detect LCA-modified cellular proteins and exhibited no cross-reactivity toward native cellular proteins, thus providing a powerful tool for capture of protein-bound LCA formed in the liver.

Analysis of protein-bound LCA in the liver of the bile duct-ligated rat and its structural analysis by MALDI-TOF-MS

To identify proteins that had formed LCA adducts, we investigated liver tissue samples obtained from the bile duct-ligated rat as an animal model simulating cholestatic liver disease. In this study, subcellular fractions obtained by

TABLE 4. Calculated and observed ions of trypsin digests of reduced and alkylated proteins in spots 2

Protein Name	Peak Number	Amino Acid Sequence From	To	Sequence	$[M+H]^+$ Calculated	Observed
					m/z	
Rab-3	1	84	93	YRTITTAYYR	1,307.7	1,308.8
(43.8)[d]	2	122	136	TYSWDNAQVILVGNK	1,707.9	1,708.8
	3	63	70	TVYR[a]HDK[a]R/VYR[a]HDKR[a] or TVYRHDK[a]R[a]	1,791.2	1,792.5
	4	73	85	LQIWDTAGQER[a]YR or LQIWDTAGQERYR[a]	1,994.1	1,994.1
	5	84	93	YR[a]TITTAYYR[a]	2,024.2	2,023.7
	6	42	60	YADDSFTPAFVSTVGIDFK	2,080.0	2,081.4
	7	187	219	M[b]NESLEPSSSPGSNGKGPALGDTPPPQPSSC[c]GC[c]	3,315.4	3,313.5
Rab-16	1′	63	72	YRTITTAYYR	1,307.7	1,308.8
(46.1)[d]	2′	101	115	TYSWDNAQVILVGNK	1,707.9	1,708.8
	3′	42	49	TVYR[a]HDK[a]R or VYR[a]HDKR[a] or TVYRHDK[a]R[a]	1,791.2	1,792.5
	4′	52	64	LQIWDTAGQER[a]YR or LQIWDTAGQERYR[a]	1,994.1	1,994.1
	5′	63	72	YR[a]TITTAYYR[a]	2,024.2	2,023.7
	6′	21	39	YADDSFTPAFVSTVGIDFK	2,080.0	2,081.4
	7′	166	198	M[b]NESLEPSSSPGSNGKGPALGDTPPPQPSSC[c]GC[c]	3,315.4	3,313.5

[a] Presumed binding site of LCA.
[b] Oxidated.
[c] Carbamoylmethylated.
[d] Percent of covered amino acids.

differential centrifugation, such as nuclei, microsomes, cytosol, and mitochondria, were examined by immunoprecipitation using Ab_2. The results derived from SDS-PAGE analysis indicated the presence of the target proteins in the cytosol (data not shown). We therefore carried out experiments aimed at isolating LCA-tagged proteins in the cytosol fraction by immunoprecipitation using Ab_2 antibody. The immunoprecipitation approach was a batch adsorption followed by elution, wherein cytosolic proteins from the liver of bile duct-ligated rats were captured. Proteins in the immunoprecipitates were resolved by 2-DE, and the gels were stained with silver stain to visualize protein bands (**Fig. 5**). Spots that were present in the specific immunoprecipitates captured by the anti-LCA antibody were excised, and three spots, which were not observed in the nonspecific immunoprecipitates captured by normal rabbit IgG, were destained and enzymatically digested as described in the Methods section. The resulting peptide mixtures were analyzed by MALDI-TOF-MS operating in linear mode (**Fig. 6**). The sequence of modified and unmodified peptides best matching the experimental molecule obtained with MALDI-TOF-MS was evaluated with a peptide mass database search (http://prospector.ucsf.edu/). The *m/z* values are the monoisotopic peaks of the protonated molecule $[M+H]^+$. As depicted in **Tables 3–5**, peptide mass profiles were obtained from the database search that identified Rab-3 and M-Ras from spot 1, Rab-3 and Rab-16 from spot 2, and Rab-12, Rab-3, and Rab-16 from spot 3 with 33.5–47.1% coverage of the protein sequence. Of particular interest is the finding of Rab-3 modified with LCA from all the spots, whereby Rab-3-bound LCAs are covalently bound with different numbers and/or different binding sites. Among the ions of the identified peptides of Rab-3, ions at *m/z* 1,792.2 and 2,022.9 were the Lys-C peptides corresponding to residues 63–70 and 84–93, each with two molecules of LCA. In the case of M-Ras, the ions at 1,658.5, 1,741.7, 1,813.6, 2,097.9, 2,385.1, and 2,502.1 were the peptides corresponding to residues 148–158, 194–202, 128–138, 174–182, 136–147, 194–208, and 117–134, each with one or two molecules of LCA. It is also noted that several ions of the peptides linked to footnote [a] in Tables 4 and 5 were the peptides modified with one or two molecules of LCA. By using this strategy, three protein spots were successfully identified.

Rab proteins are Ras-like, small GTP binding proteins that regulate vesicle trafficking pathways (34–36). The formation of these proteins covalently bound with LCA may alter tethering of transport vesicles to their target membranes. This, in turn, might contribute to LCA-induced liver toxicity or promotion of cancer. Indeed, it has been reported that LCA is found as an adduct through the lysine residues with histone in colonic mucosa epithelial cells, resulting in denaturation of the DNA double strand (5, 9). Further studies on the characterization of the modified co-

TABLE 5. Calculated and observed ions of trypsin digests of reduced and alkylated proteins in spots 3

Protein Name	Peak Number	Amino Acid Sequence		Sequence	$[M+H]^+$	
		From	To		Calculated	Observed
					m/z	
Rab-12	1	81	88	ETFDDLPK[a]	1,322.7	1,321.6
(44.8)[d]	2	142	152	DNFNVDEIFLK	1,353.7	1,352.6
	3	15	25	FTDDTFC[c]EAC[c]K	1,393.5	1,394.1
	4	81	91	ETFDDLPKWM[b]K	1,425.7	1,427.0
	5	126	134	FAQQITGM[b]R[a]	1,425.8	1,427.0
	6	80	88	K[a]ETFDDLPK or KETFDDLPK[a]	1,450.8	1,451.2
	7	96	109	YASEDAELLLAGNK	1,493.7	1,494.5
	8	117	125	EISR[a]QQGEK[a]	1,791.1	1,792.5
	9	96	116	YASEDAELLLAGNKLDCETDR	2,383.1	2,384.9
	10	153	167	LVDDILK[a]K[a]M[b]PLDVLR or LVDDILK[a]KM[b]PLDVLR[a] or LVDDILKK[a]M[b]PLDVLR[a]	2,500.6	2,502.2
	11	96	120	YASEDAELLLAGNK[a]LDC[c]ETDREISR or YASEDAELLLAGNKLDC[c]ETDR[a]EISR or YASEDAELLLAGNKLDC[c]ETDREISR[a]	3,226.7	3,224.9
Rab-3	1′	84	93	YRTITTAYYR	1,307.7	1,308.7
(44.7)[d]	2′	153	167	LAGDLGFEFFEASAK	1,601.8	1,602.2
	3′	122	136	TYSWDNAQVILVGNK	1,707.9	1,707.5
	4′	63	70	TVYR[a]HDK[a]R or TVYR[a]HDKR[a] or TVYRHDK[a]R[a]	1,791.2	1,792.5
	5′	25	41	LLLIGNSSVGK[a]TSFLFR or LLLIGNSSVGKTSFLFR[a]	2,210.4	2,212.4
	6′	187	219	M[b]NESLEPSSSPGSNGKGPSLGDTPPPQPSSC[c]GC[c]	3,315.4	3,314.3
Rab-16	1″	63	72	YRTITTAYYR	1,307.7	1,308.6
(47.1)[d]	2″	132	146	LAGDLGFEFFEASAK	1,601.8	1,602.2
	3″	101	115	TYSWDNAQVILVGNK	1,707.9	1,707.7
	4″	42	49	TVYR[a]HDK[a]R or TVYR[a]HDKR[a] or TVYRHDK[a]R[a]	1,791.2	1,792.5
	5″	4	20	LLLIGNSSVGK[a]TSFLFR or LLLIGNSSVGKTSFLFR[a]	2,210.4	2,212.4
	6″	166	198	M[b]NESLEPSSSPGSNGKGPSLGDTPPPQPSSC[c]GC[c]	3,315.4	3,314.3

[a] Presumed binding site of LCA.
[b] Oxidated.
[c] Carbamoylmethylated.
[d] Percent of covered amino acids.

lonic histones by immunoprecipitation followed by LC/MS analysis are now being conducted in our laboratory.

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