Conserved Residues in the Putative Catalytic Triad of Human Bile Acid Coenzyme A:Amino Acid *N*-Acyltransferase*

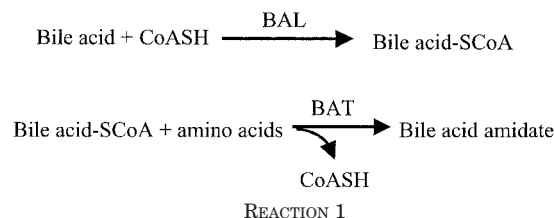## Mindan K. Sfakianos‡, Landon Wilson§, Michael Sakalian¶‖, Charles N. Falany‡, and Stephen Barnes‡§**

From the ‡Department of Pharmacology and Toxicology, the ¶Department of Microbiology, and the §Comprehensive Cancer Center Mass Spectrometry Shared Facility, University of Alabama at Birmingham, Birmingham, Alabama 35294

Human bile acid-CoA:amino acid *N*-acyltransferase (hBAT), an enzyme catalyzing the conjugation of bile acids with the amino acids glycine or taurine has significant sequence homology with dienelactone hydrolases and other α/β hydrolases. These enzymes have a conserved catalytic triad that maps onto the mammalian BATs at residues Cys-235, Asp-328, and His-362 of the human sequence, albeit that the hydrolases contain a serine instead of a cysteine. In the present study, the function of the putative catalytic triad of hBAT was examined by chemical modification with the cysteine alkylating reagent *N*-ethylmaleimide (NEM) and by site-directed mutagenesis of the triad residues followed by enzymology studies of mutant and wild-type hBATs. Treatment with NEM caused inactivation of wild-type hBAT. However, preincubation of wild-type hBAT with the substrate cholyl-CoA before NEM treatment prevented loss of *N*-acyltransferase activity. Substitution of His-362 or Asp-328 with alanine results in inactivation of hBAT. Although substitution of Cys-235 with serine generated an hBAT mutant with lower *N*-acyltransferase activity, it substantially increased the bile acid-CoA thioesterase activity compared with wild type. In summary, data from this study support the existence of an essential catalytic triad within hBAT consisting of Cys-235, His-362, and Asp-328 with Cys-235 serving as the probable nucleophile and thus the site of covalent attachment of the bile acid molecule.

In humans, the majority of bile acids (BAs)[1] synthesized by the liver are conjugated with glycine or taurine, a reaction that favors their excretion into bile (1, 2) and uptake from portal blood into the liver (3). Conjugation also promotes absorption of fat and fat-soluble vitamins A, D, E, and K in the acidic environment of the small intestine by lowering the pK_a of bile acids and hence maintaining BA solubility. Conjugation of bile acids with amino acids occurs in two steps. In the first, BAs form a thioester with CoA, catalyzed by BA-CoA ligase. In the second step, or amidation reaction, either glycine or taurine is conjugated to BA-CoA to form a BA amidate and CoA is displaced. The amidation reaction (Reaction 1) is catalyzed by bile acid CoA:amino acid *N*-acyltransferase (BAT). BAT has been purified from the livers of rats (4), cows (5), chickens (6), and humans (7, 8). The human (9) and mouse BAT (10) genes have been cloned, and the enzymatically active recombinant enzymes expressed in bacteria. A putative rat BAT (Kan-1) has been described (11); however, the expression and enzymatic characterization of Kan-1 have not been reported.

$$\text{Bile acid} + \text{CoASH} \xrightarrow{\text{BAL}} \text{Bile acid-SCoA}$$

$$\text{Bile acid-SCoA} + \text{amino acids} \xrightarrow[\text{CoASH}]{\text{BAT}} \text{Bile acid amidate}$$

REACTION 1

Sequence similarity searches at the NCBI BLAST server (www.ncbi.nlm.nih.gov/BLAST/) against non-redundant protein data base using the iterative algorithm PSI-BLAST (12) have detected significant similarity of BAT to dienelactone hydrolase and other α/β hydrolases of known structure. The α/β hydrolase fold is shared by several enzymes that apparently have diverged from a common ancestor. Despite their different catalytic functions, these enzymes all contain a conserved nucleophile-histidine-acid catalytic triad with the histidine being a completely conserved amino acid and the nucleophile and acid loops accommodating more than one type of amino acid (13). Amino acid sequence alignments also revealed that hBAT and other BATs have high homology (above or equal to 40%) with peroxisomal, mitochondrial, and cytosolic long chain acyl-CoA thioesterases or acyl-CoA thioesterases. The conserved amino acid residues are indicated in Fig. 1. Using mutation analysis, Huhtinen *et al.* established the serine-histidine-aspartic acid triad in CTE-I (14). The importance of these residues had been suggested by previous site-directed mutagenesis experiments, and the enzyme reaction mechanism speculated (15–17). Furthermore, when the active site-serine was substituted with a cysteine the thioesterase II, a fatty acyl-thioester hydrolase, was converted to an acyltransferase (18). Thus, we have hypothesized that hBAT utilizes a catalytic triad com-

[1] The abbreviations used are: BA(s), bile acid(s); BAT, bile acid CoA: amino acid *N*-acyltransferase; DTT, dithiothreitol; ESI-MS, electrospray ionization-mass spectrometry; LC-ESI-MS-MRM, liquid chromatography-ESI-MS-multiple reaction monitoring; MALDI-TOF, matrix-assisted laser desorption ionization/time-of-flight; IPTG, isopropyl-1-thio-β-D-galactopyranoside.

```
                                                                      *
 1  65% SLLASHGFATLALAYWGYDDLPSRLEKVDLEYFEEGVEFLLRHPKVLGPGVGILSVCIGA 238
 2  63% SLLASRGFATLALAYWNYDDLPSRLEKVDLEYFEEGVEFLLRHPKVLGPGVGILSVCIGA 237
 3 100% SLLASRGFASLALAHNYEDLPRKPEVTDLEYFEEAANFLLRHPKVFGSGVGVVSVCQGV 238
 4  40% SLLAGKGFAVMALAYYNYDDLPKTMETMRIEYFEEAVNYLRGHPEVKGPGIGLLGISKGG 276
 5  40% SLLAGKGFAVMALAYYNYDDLPKSIETMHMEYFEEAVNYLRSHPEVKGPGIGLLGISKGG 276
 6  40% SLLAGKGFAVMALAYYNYDDLPKTMETMRIEYFEEAVNYLRGHPEVKGPGIGLLGISKGG 235
 7  40% SLLAGKGFAVMALAYYNYDDLPKTMETMRIEYFEEAVNYLRGHPEVKGPGIGLLGISKGG 235
 8  40% SLLAGKGFAVMALAYYNYDDLPKNMETMHMEYFEEAVNYLRSHPEVKGPGIGLLGISKGG 235
 9  43% SLLAGKGFAVMALAYYNYEDLPKTMETLHLEYFEEAMNYLLSHPEVKGPGVGLLGISKGG 235
10  40% SLLAGKGFAVMALAYYNNYEDLPKDMDIIHLEYFEEAVTYLLSHPQVTGSGVGVLGISKGG 246
11  25% ALLASHGYPSLALAYFGMDTLPATLNEIPLEYFEKAIDWIITKGDISPNNLTVFGRSRGG 253
12  26% AMLASEGFVVICVAFFQFKDLPYKLEDVDVEYFLKPIEFVLGLSYTTN-MLGIQGVSFGA 243
13  25% KPFAEQGYAVLALSYFAAPGLPATAEELPLEYFDRAVAWLAAQPSVDPKAIGVYGVSKGA 138
```

```
 1  EIGLSMAINLKQITATVLINGPNFVSSN----------PHVYRGKVFQPTPCSEEFVTTN 288
 2  EIGLSMAINLKQIRATVLINGPNFVSQS----------PHVYHGQVYPPVPSNEEFVVTN 287
 3  QIGLSMAIYLKQVTATVLINGTNFPFGI----------PQVYHGQIHQPLPHSAQLISTN 288
 4  ELGLAMASFLKGITAAVVINGSVAAVGN----------TVCYKDETIPPVSLLRDKVKMT 326
 5  ELGLAMASFLKGITAAVVINGSVAAVGN----------TISYKDETIPPVSLVRNQVKMT 326
 6  ELGLAMASFLKGITAAVVINGSVAAVGN----------TICYKDETIPPVTILRNQVKMT 285
 7  ELGLAMASFLKGITAAVVINGSVAAVGN----------TICYKDETIPPVTILRNQVKMT 285
 8  ELGLAMASFLKGITAAVVINGSVAAVGN----------TISYKDETIPPVTILRNQVKMT 285
 9  ELCLSMASFLKGITAAVVINGSVANVGG----------TLRYKGETLPPVGVNRNRIKVT 285
10  ELGFAMASFLKNITAAVIINGSISNIGG----------NLQYKDETVPSVGINTKRVKRT 296
11  ELALLLGSTFKKITSVIAVAASPILFGTNTTK----LSSWKYKGEDIPYLNLKINIISPL 309
12  TIVDLLSTRYPQIKAVVSINGPHAQCSYS---------LLKEHGKQMIVPELDDSKLYFL 294
13  EASLLVASRNPALKAAGAGVGTSFVWQGISTTGGAAKSSWTLAGKPVDYLPYGPGVRFDP 198
```

```
                                                                      *
 1  ALGLVEFYR----------TFEETAD-KDSKYCFPIEKAHGHFLFVVGEDDKNLNSKVHA 337
 2  ALGLVEFYR----------TFQETAD-KDSKYCFPIEKAHGHFLFVVGEDDKNLNSKVHA 336
 3  ALGLLELYR----------TFETTQV-GASQYLFPIEEAQGQFLFIVGEGDKTINSKAHA 337
 4  KDGLLDVVE----------ALQSPL--VDKKSFIPVERSDTTFLFLVGQDDHNWKSEFYA 374
 5  KDGLLDVVE----------ALQSPL--VDKKSFIPVERSDTTFLFLVGQDDHNWKSEFYA 374
 6  KDGLKDVVD----------ALQSPL--VEQKSFIPVERSDTTFLFLVGQDDHNWKSEFYA 333
 7  KDGLKDVVD----------ALQSPL--VEQKSFIPVERSDTTFLFLVGQDDHNWKSEFYA 333
 8  KDGLKDVVD----------ALQSPL--VDKKSFIPVERSDTTFLFLVGQDDHNWKSEFYA 333
 9  KDGYADIVD----------VLNSPLEGPDQKSFIPVERAESTFLFLVGQDDHNWKSEFYA 335
10  KDGLKDIVD----------LLNNPLEGPDQKSLIPVERSDTAFLFLVGQDDHNWKSEFYA 346
11  KNALTDKLHGRTINFSKLYTNAIKNPDAIETYMIPIENTNGSILMLSGDNDHIWPSSKLC 369
12  NTILVTAPC---------FKTLTPILTEENSIPWHWIPKETAPRLIGSVDDLCAPSIHSN 345
13  SNYMAFIYG--------LYDTGLKAADAHPQAAIPVEKIHGPVMLISGRADAMWSSSAMS 250
```

```
                                                                      *
 1  KQAIAQLMKSGKK-NWTLLSYPGAGHLIEPPYSPLCSASRMPFVIPSINWGGEVIPH-AA 395
 2  NQAIAQLMKNGKK-NWTLLSYPGAGHLIEPPYTPLCQASRMPILIPSLSWGGEVIPHSQA 395
 3  EQAIGQLKRHGKN-NWTLLSYPGAGHLIEPPYSPLCCASTTHDLR--LHWGGEVIPH-AA 393
 4  REASKRLQAHGKE-KPQIICYPEAGHYIEPPYFPLCSAGMHLLVGANITFGGEPKPH-SV 432
 5  DEISKRLQAHGKE-KPQIICYPAAGHYIEPPYFPLCSAGMHLLVGANITFGGEPRAH-AV 432
 6  NEISKRLQAHGKE-KPQIICYPEAGHYIEPPYFPLCSAGMHLLVGANITFGGEPKPH-SV 391
 7  NEISKRLQAHGKE-KPQIICYPEAGHYIEPPYFPLCSAGMHLLVGANITFGGEPKPH-SV 391
 8  DEISKRLQAHGKE-KPQIICYPAAGHYIEPPYFPLCSAGMHLLVGANITFGGEPKPH-AM 391
 9  NEACKRLQAHGRR-KPQIICYPETGHYIEPPYFPLCRASLHALVGSPIIWGGEPRAH-AM 393
10  REASKRLQAHGKE-KPQIICYPETGHHIEPPYFPLCKASLNSLVGGPVIWGGEPRAH-AM 404
11  KLGIERLKQHNFS-FYYDFLEYKAGHHIYFPYQPIYKY------------NGSNENMAI 415
12  LHIQKKLQETG---HYVELELVNGGHIMEPPYYFPHHDIVYAKPQGFYCGYGGEIVLH-AK 401
13  DAVVARLKAKGFAHKVSHLAYPDAGHTAGMPALMGGSDK-----GADEAVGGTVEGN-RF 304
```

FIG. 1. **Multiple sequence alignment of hBAT and related proteins.** The multiple sequence alignment was performed using ClustalW (EMBL, European Bioinformatics Institute). The percentage of overall sequence identity to hBAT is indicated at the beginning of each sequence. The hypothesized catalytic triad composed of cysteine (or serine), aspartate, and histidine is indicated in *bold* and by the *asterisks*. *1*, kan-1, putative rBAT (gi:604902); *2*, mBAT (gi:2130558); *3*, hBAT (gi:4502351); *4*, mitochondrial acyl coenzyme A thioester hydrolase, (MTE-I) (gi:6166586) (*Rattus norvegicus*); *5*, mitochondrial acyl-CoA thioesterase 1, (MTE-1) (gi:19527402) (*Mus musculus*); *6*, cytosolic acylcoenzyme A thioester hydrolase, (CTE-I) (gi:6166127) (*Rattus norvegicus*); *7*, liver cytosolic long chain acyl-CoA thioesterase (gi:2832737) (*Rattus norvegicus*); *8*, cytosolic acyl-CoA thioesterase (gi:6753550) (*Mus musculus*); *9*, peroxisomal long chain acyl-CoA thioesterase (gi: 13375614) (*Homo sapiens*); *10*, peroxisomal acyl-CoA thioesterase 2A (gi:19527406) (*Mus musculus*); *11*, acyl-CoA thioesterase 1 (gi: 15896648) (*clostridium acet*); *12*, contains similarity to Pfam domain: PF01738 (dienelactone hydrolase family) (gi:3874660) (*Caenorhabditis elegans*); *13*, dienelactone hydrolase family protein (gi:13422143) (*Caulobacter crescentus*).

posed of Cys-235, His-362, and Asp-328 with Cys-235 as the nucleophile.

In this report we describe experiments to support the hypothesis that Cys-235, His-362, and Asp-328 are essential for the *N*-acyltransferase activity of hBAT and form the catalytic triad with Cys-235 serving as the nucleophile. Replacement of Cys-235 by serine converted hBAT from an *N*-acyltransferase into a bile acid-CoA thioesterase, which is consistent with a reaction mechanism based on the formation of a bile acid-hBAT covalent intermediate via a thioester bond. These results also demonstrate the close relationship between acyl-CoA thioesterases and *N*-acyltransferases.

EXPERIMENTAL PROCEDURES
Materials

Cholic acid was purchased from Sigma. NEM was from Research Organics (Cleveland, OH). The QuikChange site-directed mutagenesis kit was obtained from Stratagene (La Jolla, CA). Oligonucleotides were synthesized by Sigma-Genosys (The Woodlands, Texas). [2-^{3}H]Taurine (29 Ci/mmol) and [2-^{3}H]glycine (10 Ci/mmol) were purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO) and Amersham Biosciences, respectively. QIAquick Gel Extraction Kit and QIAprep Spin Miniprep Kit were purchased from Qiagen (Valencia, CA). Centricon Centrifugal Filter Devices were obtained from Millipore (Bedford, MA). Restriction enzymes were purchased from New England Biolabs (Beverly, MA) and Promega (Madison, WI). Sep-Pak Plus C_{18} cartridges were purchased from Waters (Milford, MA). pET-21a(+) and Bugbuster protein extraction reagent were obtained from Novagen (Madison, WI). The Rapid Ligation Kit was from Roche Molecular Biochemicals. SoftLink Avidin Resin was purchased from Promega.

Chemical Modification of hBAT

Time-course of NEM Modification—Bacterial cytosol containing wild-type hBAT (150 μg of total protein) was incubated with NEM (680 μM) for 2, 5, and 10 min at 37 °C. At the end of the incubations, dithiothreitol (DTT; 680 μM) was added to inactivate residual NEM. *N*-acyltransferase activity was then determined following addition of cholyl-CoA (0.5 mM), [2-^{3}H]taurine (0.1 mM) and phosphate buffer (100 mM K_2HPO_4, pH 8.25) and incubation at 37 °C for 30 min. The total reaction volume was 100 μl.

Concentration Curve of NEM Modification—Different concentrations of NEM (50, 100, 200, 400, 600, and 800 μM) were incubated with bacterial cytosol containing wild-type hBAT (150 μg of total protein) for 10 min at 37 °C. *N*-Acyltransferase activity was then determined as previously described.

Cholyl-CoA Protection of hBAT

Bacterial cytosol containing wild-type hBAT (150 μg of total protein) was preincubated with cholyl-CoA (1 mM) in phosphate buffer for 0, 2, and 5 min at 37 °C, and then NEM (680 μM) was added. The mixture was incubated for a further 10 min at 37 °C followed by the addition of DTT (680 μM). *N*-Acyltransferase activity was determined with the addition of [2-^{3}H]taurine (0.05 μCi, 0.1 mM). The total reaction volume was 120.5 μl.

Cholyl-CoA Synthesis

Cholyl-CoA was chemically synthesized as described previously (19), using a modification of the method of Shah and Staple (20). Cholyl-CoA was first separated from the reaction mixture by three extractions with five reaction volumes of ether in a separating funnel. The aqueous phase was collected and purified by absorption onto Sep-Pak Plus C_{18} cartridges. After the sample was loaded, the cartridge was washed with 3×3 ml of 20% methanol in double distilled H_2O. Elution was carried out with 10 mM NH_4HCO_3 in methanol. After removal of the elution buffer by evaporation into air, cholyl-CoA was dissolved in doubledistilled H_2O, and its concentration determined spectrophotometrically. An $\epsilon_{259\ nm} = 16,800$ M^{-1} cm^{-1} was used to calculate the concentration. Since it was difficult to detect low levels of cholate contamination in cholyl-CoA by high pressure liquid chromatography-based procedures, electrospray ionization-mass spectrometry (ESI-MS) was used to confirm the purity of the synthesized cholyl-CoA. Analysis was carried out on a PE-Sciex (Concord, Ontario, Canada) API III triple quadrupole mass spectrometer. The sample was introduced into the mass spectrometer via the electrospray ionization interface with an ionizing needle voltage of −4900 V and an orifice potential of −60 V. Negative ion

spectra were recorded over a range of *m/z* values from 190 to 2000. The purity of the cholyl-CoA preparation by this analysis was estimated to be better than 99.5%.

Generations of hBAT Constructs and Mutants

Construct hBAT Expression Vectors—The full-length cDNA for hBAT was cloned into the pKK233–2 vector as described previously (9). Plasmid pET-21a(+)/hBAT was constructed by first amplifying hBAT coding sequence by PCR with pKK233–2/hBAT as template DNA. Restriction enzyme sites *Nde*I and *Xho*I were generated at the 5′ and 3′ end, respectively. The PCR products were then digested with restriction enzymes *Nde*I and *Xho*I. The resulting fragment, containing the hBAT coding sequence, was then ligated into the similarly digested pETGagbiotinHis. The plasmid pETGagbiotinHis is a modified version of pET-21a(+) containing the coding sequence for a viral *gag* protein and a biotinylation tag.[2] This peptide tag serves as the substrate for site-specific biotinylation by the biotin ligase BirA (21). Digestion with *Nde*I and *Xho*I excised the *gag* sequence leaving behind the biotinylation tag coding sequence to be fused to the 3′ end of the hBAT coding sequence.

Site-directed Mutagenesis—A QuikChange site-directed mutagenesis kit was used for all the mutations in this study. The expression plasmid pKK233–2/hBAT or pET-21a(+)/hBAT was used as the template for PCR. Oligonucleotides used for the mutagenesis are shown in Table I with the altered nucleotides *underlined*. Following PCR, the parental DNA template was removed by *Dpn*I endonuclease digestion and the remaining PCR products transformed into *Escherichia coli* XL1-Blue cells. Colonies were selected and characterized by DNA sequence analysis, and each plasmid contained the correct mutation.

Expression of hBAT and Preparation of Cell Extracts

Plasmids were transformed into competent *E. coli* XL1-Blue or BL21 cells (containing the BirA expression vector birA/pACYC184). The cells were grown in 400 ml of Luria broth containing ampicillin (100 μg/ml), or for expression of biotinylated enzyme both ampicillin (100 μg/ml) and chloramphenicol (10 μg/ml) in a 2-liter flask at 37 °C with shaking. Isopropyl thio-β-D-galactoside was added to a final concentration of 1 mM or 0.4 mM along with biotin (50 μM in dimethyl sulfoxide) when the cells had grown to an A_{600} of ~0.6. The incubation temperature was decreased to 30 °C, and cells were harvested 4 h later by centrifugation at 3000 $\times$ *g* for 20 min at 4 °C. The pellets were resuspended in Bugbuster protein extraction reagent using 5 ml of BugBuster reagent per gram of wet cell paste and 1 μl (25 units) of Benzonase per ml of the resuspension. The cell suspension was incubated on a shaking platform at a slow setting for 20 min at room temperature. The insoluble cell debris was removed by centrifugation at 16,000 $\times$ *g* for 20 min at 4 °C. The supernatant fraction was assayed for *N*-acyltransferase activity and stored at –70 °C.

Purification of hBAT

Prepared cell extract (25 ml) was mixed with an ATP regeneration system composed of creatine phosphate (80 mM), creatine phosphokinase (500 μg/ml), and ATP (5 mM) and then loaded onto a Softlink avidin column (3 ml bed volume) equilibrated with Bugbuster protein extraction reagent (24 ml). The ATP regeneration system was used to separate hBAT from the GroEL-hBAT complex since bacterial GroEL binding to hBAT was always a problem for obtaining pure hBAT (data not shown). The column was washed with 50 mM Tris-Cl (pH 8, 15 ml). The target protein was eluted with 10 mM biotin in 50 mM Tris-Cl (pH 8, 6 ml). The column was allowed to incubate for 15 min before 1-ml fractions were collected. Protein concentration was determined by the Bradford protein assay using bovine serum albumin as the standard (22).

Enzyme Activity Determinations

Radioassay of N-Acyltransferase Activity—The *N*-acyltransferase activity assay monitors the formation of bile acid-^{3}H-amino acid conjugates (19). The standard reaction mixture contained cholyl-CoA (0.25 mM), [2-^{3}H]taurine or [2-^{3}H]glycine (0.1 μCi, 2.5 mM), and K_2HPO_4 (100 mM), pH 8.25. The reaction was started by the addition of enzyme preparation to the reaction mixture and continued for 30 min at 37 °C. The reaction was terminated by the addition of KH_2PO_4 (100 mM) pH 2.0, containing 1% SDS and n-butanol (19). The reaction products were separated from unreacted taurine or glycine by extraction into the n-butanol phase. Aliquots of the butanol phase were mixed with scintillation
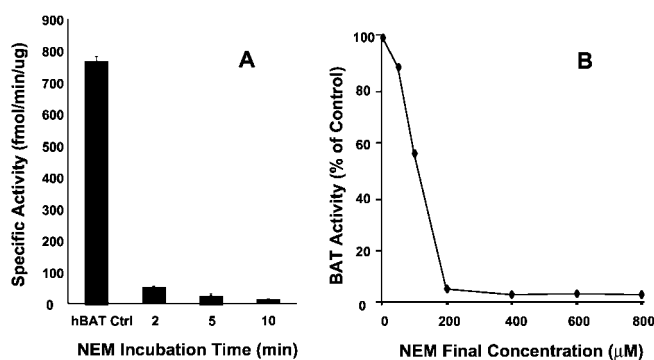
FIG. 2. **NEM modification of hBAT.** *A*, bacterial cytosol containing wild-type hBAT was incubated with *N*-ethylmaleimide in different time points at 37 °C. In 10 min, more than 90% of *N*-acyltransferase activity was lost. *B*, bacterial cytosol containing wild-type hBAT was incubated with different concentrations of NEM at 37 °C. More than 90% of hBAT *N*-acyltransferase activity was lost when NEM final concentration was 200 μM.

mixture, and the radioactivity determined in a Packard (Downers Grove, IL) 1900CA liquid scintillation counter.

Spectrophotometric Analysis of Thioesterase Activity for C235S Mutant hBAT—The assay was based on the method described previously (14). The buffer contained 200 mM potassium chloride, 10 mM Hepes, and 0.05 mM DTNB (5, 5′-dithiobis-(2-nitrobenzoic acid)). Substrates cholyl-CoA and glycine were mixed with the buffer prior to enzyme addition. The total reaction volume was 1 ml. Thioesterase activity was measured by the increasing absorbance at 412 nm.

Liquid Chromatography-Electrospray Ionization-Mass Spectrometry-Multiple Reaction Monitoring (LC-ESI-MS-MRM) Assay for N-Acyltransferase and Thioesterase Activities—Cholyl-CoA (50 μM) and glycine or taurine (2.5 mM) were incubated with wild-type or mutant hBAT (1 μg) in K_2HPO_4 (100 mM), pH 8.25, at 37 °C for 5 min. The total reaction volume was 100 μl. The reaction products were extracted with 100 μl of n-butanol by vortexing for 1 min and quantitated by LC-ESI-MS-MRM. An online reverse phase column (RP-300 C_8, 4.6 $\times$ 100 mm) was used. Buffer A was 10 mM ammonium acetate and buffer B was 10 mM ammonium acetate in 90% acetonitrile. The product was eluted from the column with a linear gradient of buffer B (0–100%) and introduced into the electrospray ionization interface in the negative ion mode. The parent ion/daughter ion combinations (*m/z* 407/343 for cholate; *m/z* 464/74 for glycocholate; and *m/z* 514/124 for taurocholate) were monitored to detect the formation of cholate, glycocholate, and taurocholate. A linear peak area-response curve was generated with standards containing known concentrations of cholate, glycocholate, and taurocholate. The estimated amounts of reaction products, cholate, glycocholate, and taurocholate were determined by comparison to the standard response curve. The apparent K_m and V_{max} for bile acid-CoA thioesterase activity for wild-type and C235A mutant hBAT were estimated using linear regression analysis of a Lineweaver-Burk double reciprocal plot. The results are presented as the means $\pm$ S.E. Comparisons of the K_m values and V_{max} values were carried out using a pooled *t* test.

Tryptic Peptide Finger Printing

The polypeptide bands observed in Coomassie Blue-stained, SDS-PAGE gels of affinity-purified hBAT were subjected to tryptic peptide finger printing. Following trypsin digestion and recovery of the peptides, they were evaporated to dryness and then reconstituted in 10 μl of 50% aqueous acetonitrile:5% formic acid. An aliquot (1 μl) was mixed with 9 μl of a saturated solution of α-cyano-4-hydroxycinnamic acid in 50% aqueous acetonitrile:0.1% trifluoroacetic acid. The mixture (1 μl) was spotted onto a MALDI target plate and allowed to crystallize. MALDI-TOF mass spectrometry was performed on an Applied Biosystems Voyager Elite time-of-flight mass spectrometer. The resulting mass spectrometry data was deisotoped, and significant ions submitted for analysis using the MASCOT software program (available at www.matrixscience.com).

RESULTS

Inactivation of hBAT by N-Ethylmaleimide—To investigate if cysteine residues are critical to hBAT enzyme activity, bacterial cytosol containing wild-type hBAT was incubated with
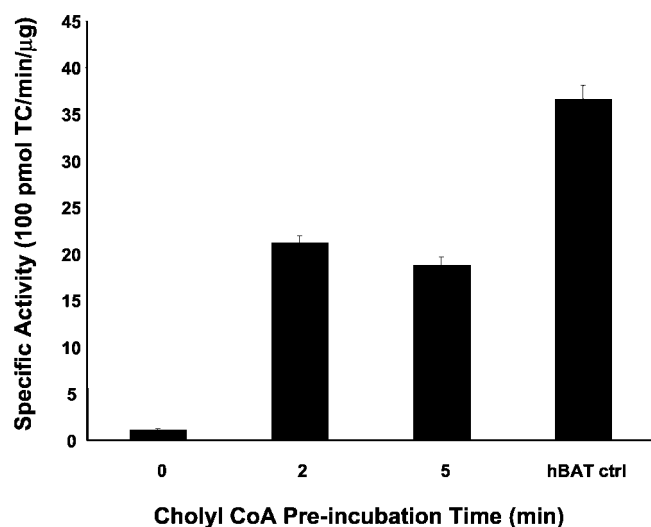
FIG. 3. **Bile acid-CoA protection of NEM inactivation.** Bacterial cytosol containing wild-type hBAT was preincubated with cholyl-CoA before NEM was added. The preincubation of cholyl-CoA with the enzyme prevented ~50% of *N*-acyltransferase activity loss.

TABLE I
Oligonucleotides used for site-directed mutagenesis to produce the C235S, H362A, and D328A hBAT mutants
The altered nucleotides are underlined.

Cys-235 → Ser (TGT→TCT)
  FP 5′-GGCGTTGGGGTAGTCTCTGTA**TCT**CAAGGAGTACAGAT GG-3′
  RP 5′-CCAATCTGTACTCCTTG**AGA**TACAGAGACTACCCCAAC GCC-3′
Asp-328 → Ala (GAT→GCT)
  FP 5′-GGAGAAGGT**GCT**AAGACTATCAACAGC-3′
  RP 5′-GCTGTTGATAGTCTT**AGC**ACCTTCTCC-3′
His-362 → Ala (CAC→GCC)
  FP 5′-GGGGCAGGC**GCC**CTGATAGAACCTCCC-3′
  RP 5′-GGGAGGTTCTATCAG**GGC**GCCTGCCCC-3′

NEM, a cysteine residue alkylation reagent, for 2, 5, and 10 min at 37 °C. DTT was then added to react with the residual NEM, and *N*-acyltransferase activities were assayed. To confirm the hBAT inactivation by NEM modification in different time periods, in a separate experiment different concentrations of NEM were incubated with bacterial cytosol containing wild-type hBAT, and *N*-acyltransferase activities were similarly determined. Fig. 2*A* shows that NEM incubation with wild-type hBAT caused a rapid decrease of *N*-acyltransferase activity. Over a 10 min period, more than 90% of activity was lost. Wild-type hBAT without NEM incubation was used as a control and had no loss of activity. Fig. 2*B* shows the effect of increasing NEM concentrations on *N*-acyltransferase activity. At 200 μM, more than 90% of the activity was lost.

Cholyl-CoA Protection of hBAT from NEM Modification— Since NEM modification caused loss of *N*-acyltransferase activity, one or more of the cysteine residues in hBAT may be critical for enzyme activity and one Cys residue could be the active site. The latter idea would be supported if the substrate, cholyl-CoA, can prevent NEM-induced loss of hBAT activity by reacting with this cysteine residue. Cholyl-CoA was preincubated with cytosol containing hBAT for 0, 2, and 5 min at 37 °C. NEM was added at the end of the incubation, and the mixture was incubated for an additional 10 min. DTT was then added to react with the residual NEM, and *N*-acyltransferase activity was assayed. Preincubation of cytosol containing hBAT with its substrate, bile acid-CoA (cholyl-CoA), before the addition of NEM preserved ~50% of BAT activity (Fig. 3).

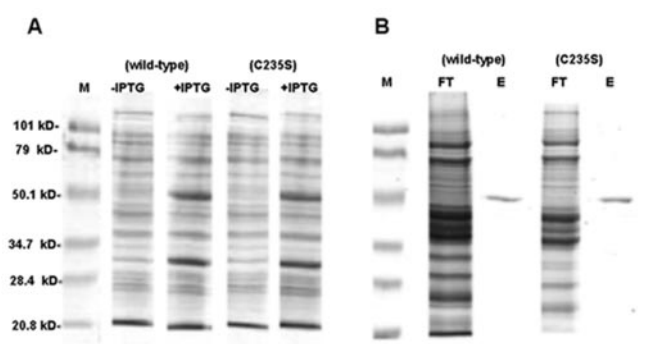*Site-directed Mutagenesis of hBAT—* As a first step to test the

FIG. 4. **Expression and purification of C235S mutant and wild-type hBAT.** *A*, expression of C235S mutant and wild type hBAT. Pellets from 1 ml of cell culture were resuspended with 5× sample loading buffer (50 μl for the pellets before IPTG addition; 100 μl for the pellets after IPTG addition). 5 μl of each resuspension was loaded onto a 10% SDS-PAGE gel. *Lane 1*, molecular mass marker; *lanes 2* and *3*, before and after IPTG induction for wild-type hBAT; *lanse 4* and *5*, before and after IPTG induction for C235S mutant hBAT. For *lanes 3* and *5*, the top bands with increased intensity are hBAT and the bottom ones are biotin ligase. Both hBAT and biotin ligase expressions were IPTG-inducible. Wild-type and C235S mutant hBATs have similar expression levels. *B*, purification of hBAT with Softlink avidin column. Cell lysate containing C235S mutant or wild-type hBAT was loaded to the column. The target protein was eluted with 10 mM biotin. 10 μl of the flow-through and 10 μl of elution fraction were loaded to a 10% SDS-PAGE gel. *Lane 1*, molecular mass marker; *lanes 2* and *3*, flow-through and eluate for wild-type hBAT; *lane 4* and *5*, flow-through and eluate for C235S mutant hBAT. hBATs were purified to almost homogeneity.
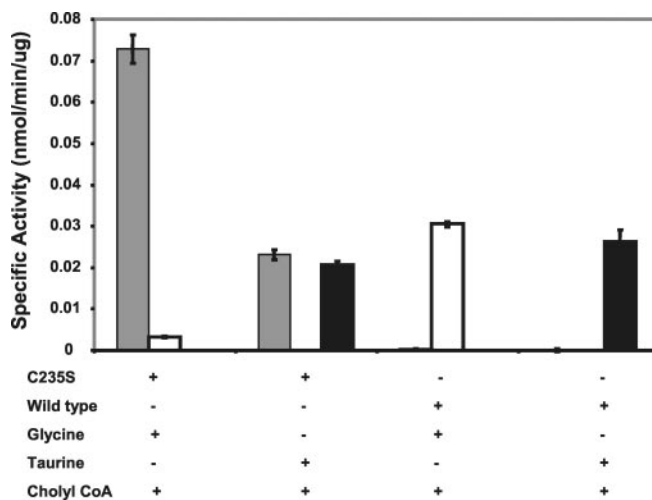

FIG. 5. **LC-ESI-MS-MRM analysis of reaction products.** Cholyl-CoA and glycine or taurine were incubated with C235S mutant or wild-type hBAT in phosphate buffer at 37 °C for 5 min. The reaction products were extracted with butanol at the end of the incubation. Aliquots of the butanol phase were used for LC-ESI-MS-MRM analysis as described under "Experimental Procedures." *Gray bars*, cholic acid; *white bars*, glycocholate; *black bars*, taurocholate. The experiments were triplicated. Cholate was the dominant product when glycine was the second substrate for C235S mutant hBAT. Very low bile acid-CoA thioesterase activity was observed for wild-type hBAT when either glycine or taurine used as the second substrate.

hypothesis that hBAT has a catalytic triad, both His-362 and Asp-328 were separately mutated to alanine. In addition, Cys-235 was mutated to a serine residue, since serine is the nucleophile for the related enzymes (Fig. 1). As shown in Table I, the codons TGT, CAC, and GAT, which code for Cys-235, His-362, and Asp-328, respectively, were changed to the codons TCT, GCC, and GCT, which code for Ser, Ala, and Ala, respectively. These hBAT mutants, along with wild-type hBAT, were expressed in bacteria. Expression levels were similar in each case. Fig. 4*A* shows the IPTG-induced overexpression of the
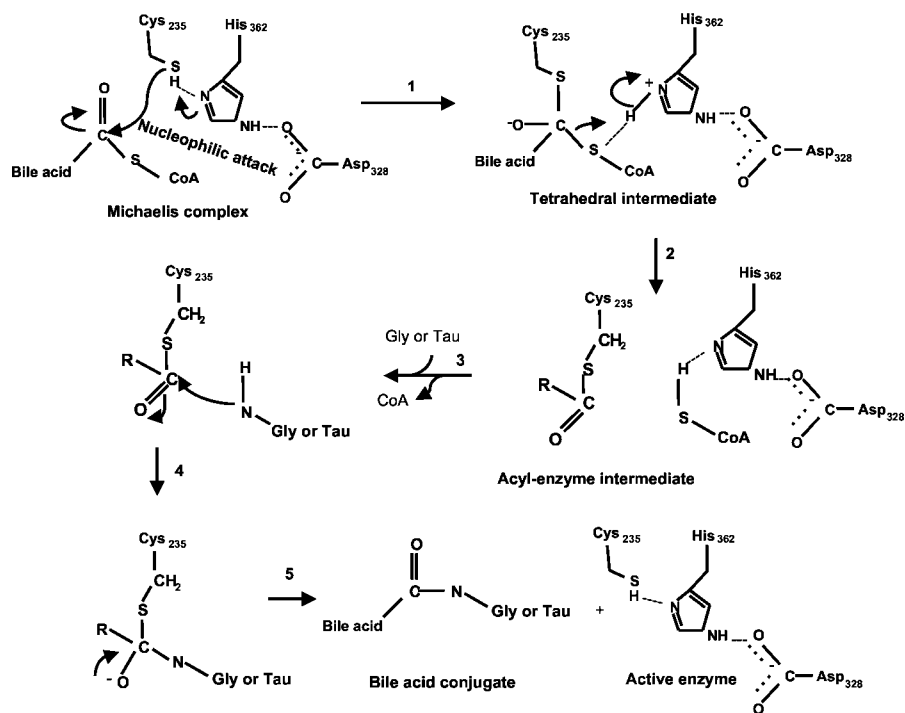
FIG. 6. **The catalytic mechanism of hBAT.** The reaction involves five steps: 1) nucleophilic attack of Cys-235 on the carbonyl carbon of bile acid-CoA substrate to form a tetrahedral intermediate; 2) decomposition of the tetrahedral intermediate to the acyl-enzyme intermediate (CoA is cleaved from bile acid-CoA); 3) amino acid (glycine or taurine) attacks the carbonyl carbon on the acyl-enzyme intermediate; 4) formation of the second tetrahedral intermediate; and 5) generation of bile acid-amino acid conjugate and recovery of the active enzyme.

C235S mutant and wild-type hBAT. In both cases, the major IPTG-induced protein bands were identified as hBAT (50 kDa) and biotin ligase (~30 kDa) by MALDI-TOF mass spectrometry analysis of their tryptic peptides. Each hBAT was purified with the Softlink avidin column to homogeneity based on SDS-PAGE (Fig. 4*B*).

Enzyme Activities of C235S, H362A, and D328A hBAT Mutants—The catalytic activities of these mutant and wild-type enzymes were tested using cholyl-CoA and taurine or glycine as the substrates. No *N*-acyltransferase activity was detected from either the H362A or D328A mutants. However, the C235S mutant displayed substantial bile acid-CoA thioesterase activity according to the spectrophotometric assay (data not shown). In ~13 min, all of the substrate was hydrolyzed based on the expected absorbance (calculated from $\epsilon = 13,600$ M^{-1} cm^{-1} for 5,5′-dithiobis-(2-nitrobenzoic acid)) (14). Since 5,5′-dithiobis-(2-nitrobenzoic acid) would inactivate wild-type hBAT by reacting with its cysteine residues (data not shown), LC-ESI-MS-MRM analysis was used for comparison of both the C235S mutant and wild-type hBAT for *N*-acyltransferase and thioesterase activities. LC-ESI-MS-MRM analysis revealed that the C235S variant of hBAT has lower *N*-acyltransferase activity compared with wild type; however, an increase in the rate of appearance of cholate, especially when glycine was used as the second substrate, was observed providing evidence of thioesterase activity (Fig. 5). The thioesterase activity of C235S hBAT was then characterized in kinetic assays. Its K_m was 27.46 ± 1.57 μM and its V_{max} was 18.58 ± 0.39 nmol/min/μg, while wild-type hBAT had a K_m of 50.02 ± 2.12 μM and V_{max} of 1.48 ± 0.04 nmol/min/μg. These differences were statistically significant ($p < 0.01$).

DISCUSSION

In the present study we have obtained evidence to support the protein modeling result and the hypothesis that hBAT utilizes a catalytic triad composed of residues Cys-235, His-362, and Asp-328 with Cys-235 acting as the nucleophile. If, indeed, hBAT uses this catalytic triad, the catalytic mechanism of hBAT may follow the classical charge-relay mechanism (Fig. 6) shared by serine proteases (23) and thioesterases proposed

by Pazirandeh *et al.* (15). The negative charge from the carboxyl ion of Asp-328 is transferred to His-362 and then to Cys-235 to enhance the power of the nucleophile. In this process, a tetrahedral intermediate **2** and one acyl-enzyme intermediate **3** are formed (Fig. 6).

Evidence for the catalytic triad, especially Cys-235 as the nucleophile, was obtained using several experimental approaches. The enzyme can be inactivated by a cysteine-specific alkylation NEM, suggesting that one or more cysteine residues are important for the enzyme activity. Cholyl-CoA preincubation before NEM addition prevented 50% of such a loss, suggesting that occupation of the critical cysteine by an excess of substrate can protect the enzyme from NEM modification.

Site-directed mutagenesis of His-362 and Asp-328 to alanine resulted in inactivation of hBAT suggesting that these two residues are critical for the enzyme activity. These results are also consistent with the conservation of these residues as shown by sequence alignment with related enzymes (Fig. 1) and the hBAT protein structure as predicted by comparison to dienelactone hydrolase and other α/β hydrolases of known structures. His-362 and Asp-328 are the only histidine and aspartate residues conserved among BATs, thioesterases, and dienelactone hydrolases (Fig. 1). Previous studies have shown that these residues are critical for enzyme activity in thioesterases and α/β hydrolases (13–18). Since the Asp and His residues are responsible for providing the nucleophile negative charge and relaying it to the cysteine residue, their replacement with alanine would destroy the charge relay system, thereby inactivating the enzyme.

When Cys-235 was mutated to a serine, the C235S mutant enzyme had a lower *N*-acyltransferase activity. In contrast, its thioesterase activity was substantially enhanced. It is thought that once an acyl-enzyme intermediate is formed, the final product formation depends on the nucleophilic attack from either one of the second substrates or water (Fig. 6). For instance, when glycine was used for the serine acyl-enzyme produced from C235S mutant hBAT, the nucleophilic attack from water is more efficient than that from glycine, resulting in cholate as the major product and glycocholate as a minor prod-

uct (Fig. 5). This can also be used to explain why β-alanine, the carboxylic acid analog of taurine, is a poor substrate of hBAT (7), whereas both 2-fluoro-β-alanine (a stronger acid) and taurine are excellent substrates (24). The result is also consistent with previous studies on thioesterases, in which the serine acyl-enzyme intermediate was shown to be unstable relative to the cysteine acyl-enzyme intermediate. For these enzymes, when the active site serine was mutated to a cysteine and the mutant enzyme was incubated with acyl-CoA, the cysteine acyl-enzyme was long-lived (14, 15). Although Cys-372 in hBAT is the other conserved Cys residue among BATs, site-directed mutagenesis suggested that despite being critical for BAT activity, it is unlikely to be the nucleophile in the enzyme reaction. When Cys-372 was mutated to an alanine residue, the C372A mutant hBAT retained low, but significant *N*-acyltransferase activity. Mass spectrometry analysis of the reaction products of C372A mutant hBAT established that this mutant hBAT retained activity (data not shown).

From amino acid sequence alignment (Fig. 1), hBAT is significantly related to acyl-CoA thioesterases. The thioesterases use a catalytic triad composed of Ser-His-Asp with Ser residue as the nucleophile. In fact, a previous study with human hepatocyte has shown that hBAT possesses some bile acid-CoA thioesterase activity and may be related to long chain acyl-CoA thioesterases (25–28). Very recently, an acyl-CoA thioesterase, PTE-2, having highest activities when CoA esters of primary bile acids cholyl-CoA and chenodeoxycholyl-CoA used as its substrates was found (29). The nucleotides that code for Cys are TGT on hBAT, and the ones for Ser are TCT on C235S mutant hBAT. Witkowski *et al.* have speculated the evolutionary significance of hydrolase to transferase conversions via single nucleotide changes in the case of a fatty acyl-thioester hydrolase, thioesterase II (18). Although this is the first time the Cys → Ser mutation has been done on the *N*-acyltransferase, the close relationship between thioesterases and hydrolases is once again demonstrated.

hBAT is one of the key enzymes for bile acid amidation, which is important for absorption of fats and fat soluble substances, such as fat soluble vitamins, in human (3). Mutations in the enzyme due to genetic defects could cause many problems because of the lack of these essential substances.

REFERENCES

1. Zouboulis-Vafiadis, I., Dumont, M., Erlinger, S. (1982) *Am. J. Physiol.* **243,** G208–G213
2. Vessey, D. A., Whitngy, J., Gollan, J. L. (1983) *Biochem. J.* **214,** 923–927
3. Schroeder, A., Eckhardt, U., Stieger, B., Tynes, R., Schteingart, C. D. Hofmann, A. F., Meier, P. J., Hagenbuch, B. (1998) *Am. J. Physiol.* **274,** G370–G375
4. Killenberg, P. G., and Jordan, J. T. (1978) *J. Biol. Chem.* **253,** 1005–1010
5. Vessey, D. A. (1979) *J. Biol. Chem.* **254,** 2059–2063
6. Czuba, B., and Vessey, D. A. (1981) *Biochem. J.* **195,** 263–266
7. Kimura, M., Okuno, E., Inada, J., Ohyama, H., and Kido, R. (1983) *Hoppe-Seyler's Z. Physiol. Chem.* **364,** 637–645
8. Johnson, M., Barnes, S., Kwakye, J., and Diasio, R. B. (1991) *J. Biol. Chem.* **266,** 10227–10233
9. Falany, C. N., Johnson, M. R., Barnes, S., and Diasio, R. B. (1994) *J. Biol. Chem.* **269,** 19375–19379
10. Falany, C. N., Fortinberry, H., Leiter, E. H., and Barnes, S. (1997) *J. Lipid Res.* **38,** 1139–1148
11. Furutani, M., Arii, S., Higashitsuji, H., Mise, M., Fukumoto, M., Takano, S., Nakayama, H., Imamura, M., and Fujita, J. (1995) *Biochem. J.* **311,** 203–208
12. Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J. (1997) *Nucleic Acids Res.* **25,** 3389–3402
13. Ollis, D. L., Cheah, E., Cygler, M., Dijkstra, B., Frolow, F., Franken, S. M., Harel, M., Remington, S. J., Silman, I., Schrag, J., Sussman, J. L., Verschueren, K. H. G., and Goldman, A. (1992) *Protein Eng.* **5,** 197–221
14. Huhtinen, K., O' Byrne, J., Lindquist, P. J. G., Contreras, J. A., and Alexson, S. E. H. (2002) *J. Biol. Chem.* **277,** 3424–3432
15. Pazirandeh, M., Chirala, S. S., and Wakil, S. J. (1991) *J. Biol. Chem.* **266,** 20946–20952
16. Witkowski, A., Naggert, J., Wessa, B., and Smith, S. (1991) *J. Biol. Chem.* **266,** 18514–18519
17. Witkowski, A., Naggert, J., Witkowska, H. E., Randhawa, Z. I., and Smith, S. (1992) *J. Biol. Chem.* **267,** 18488–18492
18. Witkowski, A., Witkowska, H. E., and Smith, S. (1994) *J. Biol. Chem.* **269,** 379–383
19. Johnson, M., Barnes, S., and Diasio, R. B. (1989) *Anal. Biochem.* **182,** 360–365
20. Shah, P. P., and Staple, E. (1968) *Steroids* **12,** 571–576
21. Beckett, D., Kovaleva, E., and Schatz, P. J. (1999) *Protein Sci.* **8,** 921–929
22. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254
23. Kraut, J. (1977) *Annu. Rev. Biochem.* **46,** 331–358
24. Sweeny, D. J., Barnes, S., Heggie, G. D., Diasio, R. B. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84,** 5439–5443
25. Solaas, K., Ulvestad, A., Söreide, O., and Kase, B. F. (2000) *J. Lipid Res.* **41,** 1154–1162
26. Lindquist, P. J., Svensson, L. T., and Alexson, S. E. H. (1998) *Eur. J. Biochem.* **251,** 631–640
27. Svensson, L. T., Alexson, S. E. H., and Hiltunen, J. K. (1995) *J. Biol. Chem.* **270,** 12177–12183
28. Svensson, L. T., Wilcke, M., and Alexson, S. E. H. (1995) *Eur. J. Biochem.* **230,** 813–820
29. Hunt, M. C., Solaas, K., Kase, B. F., and Alexson, S. E. H. (2002) *J. Biol. Chem.* **277,** 1128–1138