Molecular Identification of NAT8 as the Enzyme That Acetylates Cysteine S-Conjugates to Mercapturic Acids*§

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Our goal was to identify the reaction catalyzed by NAT8 (N-acetyltransferase 8), a putative N-acetyltransferase homologous to the enzyme (NAT8L) that produces N-acetylaspartate in brain. The almost exclusive expression of NAT8 in kidney and liver and its predicted association with the endoplasmic reticulum suggested that it was cysteinyl-S-conjugate N-acetyltransferase, the microsomal enzyme that catalyzes the last step of mercapturic acid formation. In agreement, HEK293T extracts of cells overexpressing NAT8 catalyzed the N-acetylation of S-benzyl-L-cysteine and leukotriene E4, two cysteine conjugates, but were inactive on other physiological amines or amino acids. Confocal microscopy indicated that NAT8 was associated with the endoplasmic reticulum. Neither of the two frequent single nucleotide polymorphisms found in NAT8, E104K or F143S, changed the enzymatic activity or the expression of the protein by ≥2-fold, whereas a mutation (R149K) replacing an extremely conserved arginine suppressed the activity. Sequencing of genomic DNA and EST clones corresponding to the NAT8B gene, which resulted from duplication of the NAT8 gene in the primate lineage, disclosed the systematic presence of a premature stop codon at codon 16. Furthermore, truncated NAT8B and NAT8 proteins starting from the following methionine (Met-25) showed no cysteinyl-S-conjugate N-acetyltransferase activity when transfected in HEK293T cells. Taken together, these findings indicate that NAT8 is involved in mercapturic acid formation and confirm that NAT8B is an inactive gene in humans. NAT8 homologues are found in all vertebrate genomes, where they are often encoded by multiple, tandemly repeated genes as many other genes encoding xenobiotic metabolism enzymes.

NAT8L (N-acetyltransferase 8-like) was recently identified as aspartate N-acetyltransferase, the enzyme that makes N-acetylaspartate, the second most abundant metabolite in mammalian brain (1). Aspartate N-acetyltransferase is a membrane-bound enzyme, which is inactivated by treatment with detergents and has therefore resisted various purification attempts. The approach used to determine the molecular identity of aspartate N-acetyltransferase was based on a database search for a putative mammalian protein that would have the characteristics expected for aspartate N-acetyltransferase, i.e. similarity to known N-acetyltransferases, predicted membrane association, and predominant brain expression. This search led to the selection of two candidates, one of which (NAT8L) proved experimentally to be aspartate N-acetyltransferase.

The identification of the reaction catalyzed by NAT8L raises the question of the function of the related protein NAT8, a protein with 227 residues sharing about 30% sequence identity with NAT8L. This high similarity suggests that NAT8 also acetylates an amino acid. Furthermore, NAT8 and NAT8L share a similar hydrophobic domain that most likely anchors them to membranes, and NAT8 is predicted to be associated with the endoplasmic reticulum (ER), as recently demonstrated for NAT8L (1). Finally, unlike NAT8L, which is expressed in brain, NAT8 is expressed mainly in kidney and liver (see Ref. 2 and BioGPS Atlas on line).

Because approximately a third of the enzymes that have been described until now have not yet been molecularly identified (3, 4), NAT8 may correspond to an already described enzyme. Indeed, a literature search for a membrane-bound N-acetyltransferase associated with microsomes and expressed in kidney and liver suggests that cysteinyl-conjugate N-acetyltransferase (CCNAT) (5–7) is a likely candidate. However, it should be noted that while this work was in progress NAT8 and the closely related enzyme NAT8B (renamed ATase2 and ATase1, respectively) were proposed to act as lysine acetyltransferases acting on the β-amyloid peptide-converting enzyme-1 (BACE1), a brain protease involved in the processing of β-amyloid peptide (8). This role is, however, inconsistent with the exclusive expression of NAT8 in liver and kidney (see under “Discussion”).

CCNAT belongs to the mercapturic acid pathway of xenobiotic metabolism. This pathway, which mainly occurs in liver and kidneys, is initiated by the transfer of GSH onto an

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3 The abbreviations used are: ER, endoplasmic reticulum; CCNAT, cysteine conjugate N-acetyltransferase; SNP, single nucleotide polymorphism; DTNB, 5,5’-dithiobis-2-nitrobenzoic acid; HPLC, high pressure liquid chromatography; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]1-propanesulfonic acid; LT, leukotriene; EST, expressed sequence tag.

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electrophilic acceptor (such as the insecticides ethylene dibromide and acrylamide). This step is followed either by direct excretion of the GSH conjugate from the hepatocyte into bile by glutathione conjugate transporters (MRP2, see Ref. 9) or by the hydrolysis of glutamate by the ectoenzyme \( \gamma \)-glutamyl-transpeptidase. Membrane-bound (aminooxydepeptidase M and cysteinein glycinase; see Ref. 10) or cytosolic dipeptidases (11, 12) present in kidney, liver, and other tissues can then hydrolyze the amide bond of the cysteinyl-glycine-S-conjugates. The role of CCNAT is to catalyze the acetylation of the free \( \alpha \)-amino group of the cysteine S-conjugates in liver or kidney thereby leading to the formation of mercapturic acids, which are more polar and water-soluble than the original electrophiles and are readily excreted in urine or bile by proteins of the multidrug resistance protein family (13). In the absence of acetylation, cysteinyll conjugates are unstable, undergoing \( \beta \)-elimination catalyzed by glutamine transaminase K/cysteine conjugate-\( \beta \)-lyase, with formation of toxic sulfur derivatives (10, 14). The pathway delineated above is also involved in the metabolism of the cysteinyl leukotrienes \( \mathrm{C}_4, \mathrm{D}_4, \) and \( \mathrm{E}_4 \). The latter (LTE4) is a role of NAT8 is to catalyze the acetylation of the free \( \alpha \)-amino group of the cysteine S-conjugates in liver or kidney thereby leading to the formation of mercapturic acids, which are more polar and water-soluble than the original electrophiles and are readily excreted in urine or bile by proteins of the multidrug resistance protein family (13). In the absence of acetylation, cysteinyll conjugates are unstable, undergoing \( \beta \)-elimination catalyzed by glutamine transaminase K/cysteine conjugate-\( \beta \)-lyase, with formation of toxic sulfur derivatives (10, 14). 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to calculate NAT8-CCNAT activity corresponded to the difference in $A_{412 \text{ nm}}$ measured in the presence and the absence of S-benzyl-$L$-cysteine or another substrate. S-Benzyl-$L$-cysteine stock solution (10 mM) was made in 50 mM NaOH and subsequently neutralized. Short incubation times ($\leq 10$ min) were generally used, because product formation tended to plateau at longer incubation times. We verified that the reaction rate was proportional with enzyme concentration under our assay conditions.

Radiochemical Assay of CCNAT Activity Using [acetyl-$^{3}$H]CoA—

CCNAT radiochemical assay was used mostly to measure the CCNAT activity on $\text{LTE}_4$ and occasionally S-benzyl-$L$-cysteine. The reaction was at $37^\circ$C in 0.05 ml containing 25 mM Tris, pH 7.5, 2 mM EGTA, 1 mM octyl glucoside, 100,000 cpm [acetyl-$^{3}$H]CoA, 5 or 20 $\mu$M acetyl-CoA, 0.020 or 0.175 mM $\text{LTE}_4$ and to start the reaction, 10 $\mu$l of HEK293T whole cell extract from cells transfected with either empty pEF6-HisB or pEF6-NAT8. The reaction was stopped after 60 min by addition of 0.95 ml of 10 mM Hepes, pH 7.1, with 0.1 mM DTNB and 0.1% Triton X-100. 0.05 ml of the diluted reaction mixture was used to measure total radioactivity, and 0.85 ml was loaded onto 1-ml Q-Sepharose (GE Healthcare) disposable plastic columns (Pierce), packed according to the manufacturer’s instructions, and equilibrated with 10 mM Hepes, pH 7.2, and 0.1% Triton X-100 (equilibration buffer). Noncharged or positively charged compounds were not retained and were washed with 4 ml of equilibration buffer ($\approx 10\%$ total radioactivity) followed by 8 ml of the same buffer with 0.1 M NaCl ($\approx 40\%$ total radioactivity). Before acidification of the column (pH 4), to separate [acetyl-$^{3}$H]CoA from [acetyl-$^{3}$H]CoA, the salt was removed by washing with 12 ml of equilibration buffer. Subsequently, the columns were washed with 2 ml of 0.1 M acetate, pH 4, containing 0.1% Triton X-100 followed by (3×2 ml, fraction 4) of the same buffer and by 7×2 ml of the same buffer with added 0.05 M NaCl (fraction 5). For the remaining radioactivity (50%), [acetyl-$^{3}$H]CoA was eluted from the columns with the same buffer containing 0.5 M NaCl. Fractions 4 and 5 contained [acetyl-$^{3}$H]LTE$_4$ ($\approx 2$–5$\%$ total radioactivity). NAT8 activity on LTE$_4$ was calculated using the percentage of the total radioactivity recovered in these two fractions after removal of the radioactivity corresponding to the respective blanks (reactions in the absence of LTE$_4$).

Assay of Cytotoxicity in HEK293T Cells Due to NAT8 Transfection—

Cytotoxicity was measured using CytoToxONE™ reagent (Promega) to measure lactate dehydrogenase release in the HEK293T growth media after 24 and 48 h of transfection with pEF6-HisB (control) or pEF6-NAT8 and pEF6-NAT8-E104K (active NAT8s) or pEF6-NAT8-R149K (inactivated NAT8). The assays were done according to the manufacturer’s protocol. Practically, HEK293T cells were transfected ($n = 3$) using the jetPEI transfection reagent in 24-well plates with 1 ml of cell culture media per well. After 24 and 48 h of transfection, 0.1 ml of the cell culture media was transferred to 96-well plates, compatible with the fluorometer, the temperature was equilibrated to 22°C, and the samples were mixed with 0.1 ml of CytoToxONE™ reagent. After a 5- or 10-min reaction at 22°C, the reaction was stopped by addition of 0.05 ml of the stop solution, and fluorescence was measured in a fluorescence plate reader (excitation, 544 nm; emission, 590 nm). After 24 or 48 h of transfection, total lactate dehydrogenase (100% cell lysis) was quantified by adding 0.016 ml of the lysis solution provided in the kit to each of the 24 wells containing the transfected cells with the remaining 0.9 ml of cell culture media. After mixing, lactate dehydrogenase was measured in 0.1 ml of the lysed samples as described above, and % cytotoxicity was calculated.

NAT8 Reaction Product Analysis—

Fifty $\mu$l of control or His$_{6}$-tagged NAT8-expressing HEK293T cell extract ($\approx 1.5$ mg total protein$\text{ml}^{-1}$ final concentration) was incubated in 0.3 ml of a medium containing 25 mM Tris, pH 7.5, 2 mM EGTA, 0.2 mM acetyl-CoA, and 0.25 mM S-benzyl-$L$-cysteine at 37°C. After 45 min of incubation, the reactions were stopped (5 min at 80°C), and protein was eliminated by centrifugation at 16,000 $\times$ g for 5 min at 4°C. The resulting supernatants were separated by reverse phase HPLC on a DeltaPak C18 column (Waters, Milford, MA) using a 15-min linear gradient of acetonitrile in water (0–40%) containing 0.1% trifluoroacetic acid at a flow rate of 1 ml/min$^{-1}$. The column eluent was monitored with a UV detector at 210 nm. The identity of the fraction presumably corresponding to N-acetyl-S-benzylcysteine (only found in samples containing the NAT8-expressing extract) was confirmed by mass spectrometry analysis performed on a LCQ Deca XP ion-trap spectrometer equipped with an electrospray ionization source (ThermoFinnigan, San Jose, CA). The LCQ was operated in positive mode under manual control in the Tune Plus view with default parameters and active automatic gain control. Tandem mass spectrometry analysis was done to confirm the structure of the precursor ions using low energy collision-induced dissociation with relative collision energy of 25%. Sequencing of NAT8B Gene and EST Clones—

The open reading frame of human NAT8B gene was PCR-amplified using Pfu polymerase (Fermentas) directly from genomic DNA or from plasmid DNA (IMAGE clones 1583568 LLAM4014-d1 from mixed tissues; 2586403 LLAM6447-120 from colon; 3645067 LLAM68801-k20 from ovary) using 5’- (5’-AGTGTATCATGGCTCTTATACATCACCG-3’) and 3’ (5’-CAGGGCGGC-GCGCATAGACGCCCCTGCTGAGC-3’) primers. PCR fragments were purified (PCR purification kit, Qiagen) and directly sequenced using the same primers.

Cell Culture, Transfection, and (Immunoo)fluorescence—

Chinese hamster ovary cells were propagated in Dulbecco’s modified Eagle’s medium/F-12 supplemented with 10% fetal bovine serum and antibiotics (100 units$\text{ml}^{-1}$ penicillin and 100 $\mu$g$\text{ml}^{-1}$ streptomycin). Cells were seeded at 50,000 cells$^{-2}$ in 24-well plates on glass coverslips coated with 25 $\mu$g$\text{ml}^{-1}$ fibronectin (in phosphate-buffered saline) and grown for 1 day ($\approx 80\%$ confluency). Transfection with the pEF6/Myc-NAT8 or pEF-HisB plasmid was carried out with 0.25 $\mu$g of total DNA and 0.5 $\mu$l of Lipofectamine$^{-1}$ at 37°C for 5 h followed by overnight incubation in Dulbecco’s modified Eagle’s medium, F-12, 10% fetal bovine serum without antibiotics. Co-transfection with pEF6/Myc-NAT8 and pCMV5-NAT8L (1) constructs was carried out at 0.1 and 0.5 $\mu$g of DNA$\text{cm}^{-2}$, respectively. After 24 h, cells were processed for immunofluorescence as described elsewhere (1, 20). For primary antibodies, we used mouse monoclonal antibody IgG2a anti-Myc (9B11) (1:500;...
The JTT model of evolution and a γ rate distribution with four substitution rate categories fitted best the data. To estimate Bayesian posterior probabilities, Markov Chain Monte Carlo chains were run for 54,000 generations until convergence and sampled every 100 generations (burn-in, 15,000 generations). The NAT8L sequences were used as an outgroup. Information concerning the dataset used for tree construction is shown in the legend of Fig. 8.

**RESULTS**

Comparison of NAT8 with Homologous Sequences—Fig. 1 shows an alignment of human NAT8 and NAT8B protein sequences with the following species as well as NAT8Ls from human and *Danio rerio*. Compared with NAT8L, which includes about 300 residues, NAT8 and NAT8-related sequences are shorter (≈230 amino acids), missing the extremely conserved sequence of about 20 amino acids as well as the less conserved proline- and glycine-rich regions that are both found at the N terminus of all NAT8L orthologues (1). However, NAT8 shares with NAT8Ls the following: 1) a rather conserved region of about 30 residues, presumably responsible for the attachment of N-acetyltransferase activity of translocated sequences.

**N-acyetyltransferase Reaction Catalyzed by NAT8**—To investigate the enzymatic activity of human NAT8, we transfected HEK293T cells with eukaryotic vectors allowing the expression of human NAT8 as a fusion protein either with an N-terminal His6 tag and examined with an LSM 510 META confocal microscope. Information concerning the dataset used for tree construction is shown in the legend of Fig. 8.

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FIGURE 2. Expression of NAT8 in HEK293T cells. Western blot analysis of extracts (20 μg of total protein per lane) from HEK293T cells transfected for 48 h with empty pEF6-HisB (lanes 1–3), pEF6-NAT8 (lanes 4–6), and pEF6/Myc-NAT8 (lanes 7–9). The anti-His antibody used was the penta-His antibody (Qiagen) that detects both N- and C-terminally tagged NAT8s but with different affinities. Triplicates are the result of three independent transfection experiments.

FIGURE 3. Catalytic activity of overexpressed human NAT8. The acetyltransferase activity of whole cell extracts from HEK293T cells transfected with either pEF6-NAT8 (b) or the corresponding empty plasmid (pEF6-HisB; a) and pEF6/Myc-NAT8 (d) or the corresponding empty plasmid (pEF6/Myc-HisA; c) were assayed as described under “Experimental Procedures” with 0.5 mM of the indicated substrates. 0.2 mg acetyl-CoA and ~30 μg of total protein. Results are means ± S.E. for three independent measurements performed with the same extract.

affected cell extracts was determined using a series of substrates by comparing the formation of free CoASH from acetyl-CoA in the absence and presence of these substrates. After appropriate incubation time (often 10 min at 37 °C), addition of DTNB, which rapidly reacts with free CoASH produced during the incubation period, results in a stoichiometric increase of absorbance at 412 nm. Furthermore, DTNB strongly inhibits catalytic activity of overexpressed NAT8, as found previously for NAT8L. (1). As shown in Fig. 3, cell extracts containing either N- or C-terminal His6-tagged NAT8 (b and d, respectively) catalyzed the acetylation of S-benzyl-L-cysteine, whereas no such activity was detectable in extracts of control cells transfected with empty vectors. Furthermore, NAT8 specifically acetylated the model cysteine conjugate (S-benzyl-L-cysteine), and no significant activity was detected with L-aspartate, L-lysine (or L-lysine blocked on either the α- or the ε-amino), L-cysteine, L-methionine, L-ethionine, L-cystathionine, or other amino acids (L-phenylalanine, L-tyrosine, and L-tryptophan) containing an aromatic ring structure resembling the benzyl ring in our model substrate. By contrast, human recombinant aspartate N-acetyltransferase (NAT8L) acetylated L-aspartate but did not act on the cysteine conjugate S-benzyl-L-cysteine (data not shown).

To ensure that NAT8 had catalyzed the acetylation of the amino group of its substrate, the reaction product derived from S-benzyl-L-cysteine under identical conditions was purified by HPLC and analyzed by tandem mass spectrometry. As expected for N-acetyl-S-benzyl-L-cysteine, the product had a protonated molecular ion with m/z 254, and its fragmentation pattern was entirely consistent with acetylation of the amino group in S-benzyl-L-cysteine (Fig. 4). Most particularly the presence of a fragment with m/z 195 indicated the loss of an acetamido group (49 atomic mass units).

As reported previously for aspartate N-acetyltransferase (i.e. NAT8L), the activity of NAT8 was slightly stimulated by a low concentration (1 mM) of octyl glucoside but was inhibited by higher concentration (80% inhibition at 10 mM) of this detergent. Other detergents (CHAPS and Triton X-100) were also inhibitory (data not shown). No attempt was therefore made to purify the overexpressed protein, and the kinetic studies were performed on crude cell extracts.

A study of the kinetic properties, performed on extracts containing N-terminal His6-tagged NAT8, indicated that the $K_m$ for S-benzyl-L-cysteine was 64 ± 16 μM, and the $V_{max}$ was 4.4 ± 0.3 nmol min$^{-1}$ g$^{-1}$ protein in the presence of 0.2 mM acetyl-CoA. The $K_m$ for acetyl-CoA was 23 ± 13 μM, and the $V_{max}$ was 3.1 ± 0.6 nmol min$^{-1}$ g$^{-1}$ protein in the presence of 0.5 mM S-benzyl-L-cysteine. We also tested the activity of NAT8 on LTE4, a cysteinyl-conjugate that is known to be a substrate for rat (23-25) and human (17, 26) CCNAT. Using a radiochemical assay with [acetyl-3H]CoA (details under “Experimental Procedures”), LTE4 (0.175 mM) was acetylated by cell extracts containing N-terminal His6-tagged human recombinant NAT8 at a rate of 5.3 pmol min$^{-1}$ g$^{-1}$ protein calculated after 60 min of reaction at 37 °C. This activity is about 100-fold lower than that observed with 0.5 mM S-benzyl-L-cysteine under identical conditions.
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No acetylation of LTE was observed in extracts of control cells (data not shown).

Subcellular Localization of NAT8—As CCNAT is associated with microsomes in liver and kidney (5, 6), we next analyzed the subcellular localization of NAT8. This was performed by transfection of Chinese hamster ovary cells with the N- and C-terminal His-tagged forms of NAT8 followed by double immunolabeling using anti-Myc tag antibody (C-terminal fusion) or anti-poly-His antibody for NAT8 and appropriate organelle markers. Irrespective of the tag, NAT8 immunostaining partly delineated the nuclear envelope (filled arrowheads in Fig. 5a and supplemental Fig. S1) and extended into the cytoplasm with the characteristic reticular pattern of the ER. Continuities with the nuclear envelope are indicated by two open arrowheads in Fig. 5b. Association with the ER was confirmed by partial co-localization for both NAT8 constructs with KDEL-bearing ER protein (arrows in Fig. 5a and corresponding line intensity profile in supplemental Fig. S2; arrows in supplemental Fig. S1a), which were more enriched toward the cell periphery as expected for an ER retrieval system. The observation that co-localization was not complete is in keeping with the well known heterogeneity among ER domains (27, 28). In contrast, the NAT8 signal was fully resolved from the Golgi complex, recognized by immunolabeling for GM130 (Fig. 5b and supplemental Fig. S1) or after co-transfection with the PDib-HA construct (1, 29; data not shown). There was also complete segregation from mitochondria labeled in vivo by MitoTracker red (Fig. 5c).

Because the subcellular localization of NAT8 constructs showed strong similarities with that previously described for NAT8L (1), we directly compared their pattern in the same cells. Control experiments in cells transfected with the NAT8 construct alone indicated that affinity-purified antibodies recognizing nontagged NAT8L did not cross-react with NAT8 (data not shown). NAT8-Myc and NAT8L largely, but not fully, co-localized (Fig. 5d), with NAT8L appearing more central than NAT8.

Effect of NAT8 Mutations on Its CCNAT Activity and Toxicity—Single nucleotide polymorphisms (SNPs) on NCBI databases indicate the occurrence of two rather frequent SNPs in the NAT8 gene that result in an amino acid change (E104K; F143S) in the NAT8 protein. As this gene or at least SNPs in its promoter region have been recently shown to be linked with higher systolic blood pressure and kidney failure in hypertensive patients (30), it was of interest to determine whether the two SNPs that result in an amino acid replacement affect the CCNAT activity of NAT8. Furthermore, differences in CCNAT activity in human kidney biopsies have been reported (31). As a control, we also mutated Arg-149, which is highly conserved in NAT8Ls and NAT8 homologues (see Fig. 1). Fig. 6 shows that the two SNPs either alone or in combination did not affect by more than 2-fold the CCNAT activity (Fig. 6a) and the degree of expression, as determined by Western blotting (Fig. 6b). The changes in specific activity, as corrected for expression (Fig. 6c), were minimal. By contrast, the conservative replacement of the highly conserved residue Arg-149 by a lysine almost cancelled the activity while causing little effect on the degree of expression of the protein.

While carrying out these and other transfection experiments, we noted that expression of NAT8 led to some degree of cell death. We were interested in better appreciating this phenomenon because it was previously reported (32) that when mRNAs from human, mouse, or Xenopus NAT8 homologues were injected into Xenopus embryos it resulted in toxicity and inhibition of gastrulation movements. Therefore, we quantified cytotoxicity in transfected HEK293T cells, using the CytoTox-96 cytotoxicity assay (Promega), which allowed us to measure the release of lactate dehydrogenase in the medium of cells that had been transfected with different N-terminal tagged NAT8 constructs or with an empty vector. Fig. 7 shows that expression of wild-type NAT8, the E104K mutant, and the catalytically inactive R149K mutant had significant and similar effects on the release of lactate dehydrogenase in the medium. This effect was much more apparent after 48 h than after 24 h. These findings clearly indicate that NAT8 overexpression has toxic effects.

Analysis of the NAT8B Sequence—Besides NAT8, protein databases include a closely related protein designated NAT8B, the same length as NAT8 and sharing about 90% sequence identity. The sequence of NAT8B, which is derived from EST clone IMAGE:1083919 (32), is in contradiction with the genome assemblies, which predict a premature stop codon at codon 16, where a serine is predicted from the...
sequence of Popsueva et al. (32). As this could represent a polymorphic position, we amplified the NAT8B-coding sequence from genomic DNA from 24 individuals and sequenced it. All sequences predicted a homozygous stop codon at position 16. Of note, no SNP is found at the 16th codon of NAT8B in the NCBI SNP databases. Thus, a Ser codon in this position is extremely rare if ever present in genomic DNA. As for the cDNA sequence of NAT8B, a stop codon at codon 16 was observed in all ESTs corresponding to NAT8B (three that are present in databases are as follows: BC069564.1, BC121101.1, and BC121102.1; three others present are as follows: IMAGE clones 3645067, 2586403, and 1583568, which we sequenced), the only exception being the sequence of Popsueva et al. (32). This suggests that the Ser-16 codon is an extremely rare mutation or the result of a sequencing error.

The premature stop codon of NAT8B is followed in the same reading frame by an ATG at codon 25. The protein starting from this second ATG is likely to be inactive because it is truncated from the first 24 amino acid residues, which include several residues that are conserved in NAT8 and NAT8L homologues. To verify this, we overexpressed in human embryonic kidney cells short versions of both NAT8B and NAT8 proteins that started at Met-25 and were fused with an N- or C-terminal His6 tag. The truncated NAT8s and NAT8Bs were visible in Western blots using anti-His antibodies but were less expressed than the wild-type NAT8s (data not shown). In agreement with our prediction, the extracts containing the truncated NAT8s and NAT8Bs did not detectably acetylate S-benzyl-L-cysteine in the presence of acetyl-CoA (data not shown).

Database Analysis of NAT8 Homologues in Various Species—BLAST searches indicated that homologues of NAT8L and NAT8 are found in all vertebrate genomes. One single NAT8L gene is found per genome. By contrast, the number of NAT8 homologues per genome was one (dog and macaque), two (human, Pongo, horse, and D. rerio), six (Monodelphis), seven (rat), and eight (mice). Inspection of the genome organization indicated that when several NAT8 homologues were found, they were present as tandemly repeated genes in one genomic localization, suggesting that the NAT8 gene underwent several duplication events in some lineages, whereas the NAT8L gene, although closely related in terms of structure, did not undergo this kind of evolution.
Another striking feature revealed by the sequence alignments was that NAT8 sequences were much less conserved than NAT8L. Thus, if one considers the region of NAT8L that aligns with NAT8s, there is not a single amino acid difference between the sequences of mouse and rat (100% identity), whereas the two closest NAT8 homologues of mouse and rat (CML4) show 22 differences for 222 residues (90% identity).

In the evolutionary tree of NAT8 and NAT8L sequences shown in Fig. 8, NAT8Ls are represented by a single gene in each organism and form a cluster in this tree. This orthologous group of genes is strongly separated from all other NAT8/NAT8B homologues. Its branches following vertebrate radiation are much shorter than the branches observed for the NAT8 homologues. Another remarkable feature is that the gene duplication events leading to the presence of multiple copies of NAT8 seem to have occurred in separate lineages, independently from each other. This may suggest that there is a strong selective pressure for gene duplications as has been observed for other genes encoding enzymes involved in metabolism of xenobiotics (33, 34). However, there is a more parsimonious explanation for the peculiar nature of the phylogenetic tree. Multiple gene duplications early in vertebrate evolution followed by events of homologous recombination and subsequent losses of NAT8 homologues could also have led to the observed tree topology. Indeed, a close inspection of the rat nat8 sequences has revealed clear evidence for occurrence of such recombinatorial events, where in one of them nat8B was formed by a recombination between cml1 and cml4 (see supplemental Fig. S3).

**DISCUSSION**

Identification of the Function of NAT8—Mercapturic acid synthesis is important to allow the detoxification and the excretion of cysteinyl conjugates under the form of mercapturic acids. Although most enzymes of this pathway have been molecularly identified for many years, the identity of the enzyme that catalyzes the last step was still unknown. Here, we report the identification of CCNAT as NAT8. This conclusion is based on the finding that recombinant cells overexpressing NAT8 acetylate a typical substrate of this enzyme, S-benzyl-L-cysteine, as well as LTE₄. Moreover, a mutation affecting a conserved residue (R149K) almost abolishes this enzymatic activity while barely interfering with the expression of the recombinant protein. The identification of CCNAT with NAT8 is also consistent with previous findings indicating that this enzyme is essentially expressed in kidney and in liver (2), is membrane-bound, and is associated with microsomes (5, 7).

Aignier et al. (6) purified CCNAT from pig microsomes and tentatively identified it as a 34-kDa protein, extremely prone to
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The amino acid composition reported by these authors also substantially differs from that of NAT8 and from the pig homologue that we could find by blasting a pig genomic database. Our interpretation is that CCNAT was a minor component of the purified preparation in work by Aignier et al. (6) and was not visible by SDS-PAGE analysis. It is indeed very unlikely that CCNAT would be a major constituent of liver microsomes, as would be inferred from the report that a 6-fold purification from liver microsomes was sufficient to reach homogeneity.

Role of SNPs and Toxicity of NAT8—The activity of CCNAT varies by more than 10-fold in human kidney samples (31). It was therefore of interest to determine whether two frequent SNPs that lead to the replacement of an amino acid affected the activity of NAT8. Neither of the two tested SNPs affected the activity by more than 1.5-fold, indicating that they are not involved in the large differences mentioned above. Sequence differences in the promoter region might explain the results by Altuntas and Kharasch (31). Interestingly, minor alleles of SNPs present in the promoter region of the NAT8 gene have been shown to confer a protective effect towards both elevated blood pressure and the risk of kidney failure (30). In view of the role of NAT8 as CCNAT, this protective effect may result from a higher ability to acetylate cysteine conjugates, which in the nonacetylated form are easily converted to nephrotoxic products (14, 30, 35, 36). It would therefore be of interest to investigate if these minor alleles drive a higher expression of NAT8.

We also found that NAT8 overexpression in HEK293T cells is toxic and leads to cell death. Accordingly, lactate dehydrogenase, a cytosolic enzyme, was released in the culture media of cells transfected with NAT8-expressing plasmids. This effect does not appear to be linked to the catalytic activity of this enzyme. We speculate that it results from the overexpression of a membrane protein in the ER. This toxic effect may be related to the finding that Xenopus camello (Xcm1), a Xenopus homologue of NAT8, as well as human NAT8 inhibit gastrulation when their mRNAs are injected into the dorsovegetal blastomeres of Xenopus embryos (32).

Rapid Evolution of the NAT8-related Sequences—Homologues of NAT8 are found in many genomes. They differ from NAT8L, the protein that catalyzes the synthesis of N-acetylaspartate, by a shorter sequence (≈230 residues compared with ≈300) and a lower conservation. Whereas there is only one homologue of NAT8L per genome, this number may reach 8 for NAT8. These genes are clustered in a similar region of mammalian chromosomes where they are present as tandemly repeated genes, suggesting that they arose through several segmental duplications. We believe that they all encode cysteinyl-

N-acetyltransferases, which differ in their specificity with respect to the structure of the conjugate to which cysteine is bound. Other xenobiotic metabolizing enzymes such as glucuronosyltransferases, cytochromes P450, glutathione S-transferases, and sulfortransferases are often encoded by gene clusters resulting from several rounds of segmental duplication. The comparison with the group of cytochrome P450 enzymes is particularly interesting because some of these act on endogenous compounds and others on xenobiotics. Although there is only one gene per genome in the case of enzymes that metabolize endogenous compounds, there is considerable variety in
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the number of enzymes that metabolize xenobiotics, some of them being absent in some species and present in multiple copies in other genomes (34, 37). The same situation applies to NAT8L, which serves to acetylate L-aspartate and is encoded by one single gene per genome, and NAT8, which acts on xenobiotic metabolites and is present in variable copy numbers depending on the species. The presence of multiple copies of NAT8-related sequences in some genomes strongly supports the involvement of NAT8 in the xenobiotic metabolism.

NAT8B Does Not Encode a Functional CCNAT—Our work also leads to the conclusion that the human NAT8B gene, which resulted from a duplication of the NAT8 gene, does not encode a functional protein in humans. A premature stop codon was indeed found systematically in genomic sequencing, as well as in six independently sequenced cDNAs (three in this work and three reported in the NCBI database). Furthermore, the SNP database does not indicate the presence of a SNP at codon 16, despite extensive sequencing of this region, as indicated by statistics on a neighboring SNP at codon 23. The only sequence in which codon 16 is not a stop codon is the one reported by Popsueva et al. (32). We believe that the full-length NAT8B protein does not exist in humans or is extremely rare. We verified that the shorter protein starting from the following methionine (Met-25) in the NAT8B sequence has no CCNAT activity and that this was also true for the similarly truncated NAT8. This loss of activity is ascribable to the removal of conserved residues, most particularly Arg-7 (see Fig. 1), which is strictly conserved. It should be noted that another stop codon, replacing Gln-168, and another mutation replacing the highly conserved Gly-23 by an arginine are frequent alleles of the NAT8B gene (p = 0.326 and 0.241, respectively). As dead genes rapidly accumulate mutations (38), the presence of these two alleles with high frequency in the population is consistent with NAT8B being no longer functional.

Other Catalytic Activities of NAT8 and NAT8B—While this work was in progress, Ko and Puglielli (8) reported observations supporting the idea that NAT8B and NAT8 (designated ATase1 and ATase2) are N\(^{\text{\textsuperscript{\text{-}}}}\)-acetyl-L-lysine acetyltransferases that act on BACE1, allowing its transfer to the ER Golgi intermediate compartment and Golgi apparatus. BACE1 is an aspartate protease essentially present in brain, which hydrolyzes \(\beta\)-amyloid (A\(\text{\textsuperscript{\text{\text{-}}}N}\)) precursor protein to form \(\beta\)-amyloid peptide. This led to the suggestion that ATase1 and ATase2 might be indirectly involved in the pathophysiology of Alzheimer disease.

We wish to make three remarks about this work. First, although BACE1 is effectively expressed in brain (BioGPS Atlas available on line), NAT8 (ATase2) is exclusively expressed in kidney and liver (2).\(^4\) The acetylation of BACE1 is thus an in vitro observation, which most likely has no physiological relevance.

A second consideration is that many enzymes are not totally specific for their physiological substrate, i.e. they can act with lower catalytic efficiencies on other substrates than on their “true substrate.” Therefore, it would not come as a surprise if NAT8 had a low N-acetyltransferase activity on substrates other than cysteine S-conjugates. Unfortunately, it is virtually impossible to compare the catalytic activity of NAT8/ATase2 measured on BACE1 by Ko and Puglielli (8) with the CCNAT activity that we report. Indeed, no information is available on the amounts of protein substrate (BACE1), of enzyme (ATase1 or ATase2), and of the radiolabeled acetyl-CoA used in the BACE1 acetylation assay (8). Furthermore, it is intrinsically difficult to compare the activity measured on a low molecular weight substrate, which is present at a much higher concentration than the catalyst (NAT8), with the activity determined on a protein substrate (BACE1), which is present in concentrations similar to those of the catalyst. To circumvent this problem, we checked the ability of NAT8 to acetylate both L-lysine and two L-lysine derivatives that resemble protein-bound lysines (i.e. with no ionic charge on their \(\alpha\)-amino and/or carboxylic groups). No detectable acetylation was observed with these substrates (Fig. 3), indicating that the catalytic efficiency of NAT8 on the L-lysine derivatives that we used was at least 20-fold lower than on S-benzyl-L-cysteine. This suggests that NAT8 acetylates much better S-benzyl-L-cysteine than protein-bound lysines.

Third, Ko and Puglielli (8) studied the function of a protein (ATase1, i.e. NAT8B) that does not exist in humans. The expression vector they used indeed encodes a protein in which the stop codon at position 16 has been mutated “back” to a serine residue by OriGene. In conclusion, although we cannot rule out that NAT8 can work in vitro on BACE1, its tissue distribution and its kinetic properties plead against this being its physiological function.

Subcellular Localization and Topology of NAT8—Both our work and that of Ko and Puglielli (8) indicate that NAT8 is associated with the ER. However, Ko and Puglielli (8) as well as Popsueva et al. (32) also reported an association with the Golgi, which could be due to the analysis of overtransfected cells. Indeed, in our subcellular localization experiments, we chose to select cells with a lower level of expression of NAT8, because when we used very high levels of expression (and only in this case) NAT8 could also be detected in the Golgi apparatus (data not shown). Like for NAT8L (1), the mechanism by which NAT8 is targeted to the ER remains unsolved. The absence of signal peptide indicates that these related proteins are synthesized on free ribosomes and associate secondarily with membranes. Further work is needed to identify this mechanism and in particular to clarify the likely role of the hydrophobic domain shared by NAT8 and NAT8L.

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Molecular Identification of NAT8 as the Enzyme That Acetylates Cysteine S-Conjugates to Mercapturic Acids
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