Short Communication

Substrate Selectivity of Mouse N-Acetyltransferases 1, 2, and 3 Expressed in COS-1 Cells

(Received November 7, 1997; accepted January 21, 1998)

This paper is available online at http://www.dmd.org

ABSTRACT:
Two human acetyl-CoA:arylamine N-acetyltransferases (NAT1 and NAT2) have been identified. Therapeutic and carcinogenic agents that are substrates for these isoenzymes (including isoniazid, sulfamethazine, p-aminobenzoic acid, 5-aminosalicylic acid, and 2-amino-5-fluorouracil) have been used to evaluate the role of the N-acetylation polymorphisms of NAT1 and NAT2 in the treatment of disease and differential risk of various cancers among individuals of differing acetylator phenotypes. The mouse is frequently used as a model of the human acetylator polymorphism. As three NAT isoenzymes have been identified in mouse, it is necessary to determine the selectivity of mouse Nats toward common NAT substrates. In the present study, Nat1\*, Nat2*8, and Nat3* were expressed in COS-1 cells, and their substrate selectivity was evaluated with various substrates. Under the conditions used, mouse Nat2 had 20- to 2.4-fold higher catalytic activity for p-aminobenzoic acid, 5-aminosalicylic acid, and 2-aminofluorenone, respectively, than Nat1. Isoniazid N-acetylation was catalyzed only by mouse Nat1. For the substrates tested in this study, mouse Nat3 exhibited activity only toward 5-aminosalicylic acid and only at 1/20 the activity shown by Nat2. In addition, p-aminobenzoylglutamate, the first endogenous NAT substrate identified, was selective for mouse Nat2. These results further support the functional analogy of mouse Nat2 and human NAT1.

The ability to enzymatically N-acetylate aromatic amines is a common occurrence in living organisms. Humans and other mammals (Weber, 1987) as well as fungi (Kulkarni and Sherman, 1994; Lee et al., 1988) and bacteria (Chung et al., 1997; Hasmann et al., 1986; Watanabe et al., 1994) have been shown to possess this ability. Although N-acetylation is nearly ubiquitous among species, the role of arylamine N-acetyltransferases (NATs) in the metabolism of endogenous compounds is only at the early stages of discovery (Minchin, 1995). Many drugs and other xenobiotics, including hydrazines and carcinogenic carbocyclic and heterocyclic arylamines, are substrates for these enzymes. The wide distribution of NAT in tissues (Chung et al., 1993) as well as among species (Vatsis et al., 1995), and the apparent long evolutionary history of NAT, indicate that the enzyme may have significant physiological functions.

In humans, as in several laboratory animals, N-acetylation of many compounds is subject to a genetic polymorphism that allows individuals to be classed as rapid, intermediate, or slow acetylators. In humans, the classification of acetylator phenotype has been made on the basis of the metabolism of sulfamethazine, isoniazid, dapsone, or isonicotinamide (Vatsis et al., 1997). The NAT enzyme system is polymorphic, a third Nat, Nat3, has been identified in mice (Fretland et al., 1991), so, at least for now, the mouse enzyme can be considered monomorphic. A third Nat, Nat3, has been identified in mice (Fretland et al., 1997), although less is known about its properties and substrate selectivity.

In this paper, we report the first expression of recombinant Nat3 in mammalian cells and the substrate selectivity profile of the three

Send reprint requests to: Gerald N. Levy, Department of Pharmacology, 1301 MSRBIII, 1150 W. Medical Center Drive, University of Michigan, Ann Arbor, MI 48109-0632.

This work was supported by National Institute of Health grants GM 44965 and CA 39018.

1 Abbreviations used are: NAT, N-acetyltransferase; pABG, p-aminobenzoylglutamate.

2 A consensus paper on nomenclature of N-acetyltransferases (Vatsis et al., 1995) suggests the symbol NAT for N-acetyltransferase genes. However, the long-established style for mouse genes is to capitalize the first letter of the gene symbol followed by two lower case letters. We have followed the convention for the mouse; therefore, Nat is used.

Materials and Methods

Animals. Inbred C57BL6/J (B6) and A/J (A) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Outbred CD1 mice were obtained from Charles Rivers Laboratories (Wilmington, MA). Mice were housed in the University of Michigan Medical School animal care facility, maintained at room temperature on a 12-hr light/dark cycle, and provided Purina mouse chow and tap water.

Materials. Restriction endonucleases and other DNA-modifying enzymes were purchased from New England Biolabs, Life Technologies, Inc., and Promega. All cell culture media and the lipofectamine reagent were from Life Technologies, Inc. The pZeoSV expression vector kit was obtained from Invitrogen. The p-acetamidobenzoic acid was from Eastman Organic Chemicals (Rochester, NY), and dithiothreitol, electrophoresis grade, was from Schwarz/Mann Biotech (Cleveland, OH). Other chemicals and buffers were molecular biology grade and obtained from Sigma, Acetyl-N-(p-acetamidobenzoyl)-L-glutamic acid was a gift from Dr. Minchin (University of Western Australia), and N-acetyl-5-aminosalicylic acid was obtained from Dr. Montrose (The John Hopkins University, Baltimore, MD). The oligonucleotide primers used for PCR amplification and sequencing were synthesized by The University of Michigan DNA synthesis facility.

Amplification and Direct Sequencing of PCR-Generated Nat3*. Amplification reaction mixtures (100 μl) contained 1 μg of genomic DNA, 300 ng of each amplification primer, 200 μM each of dATP, dCTP, dGTP, and dTTP, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.01% gelatin, 400 μM dithiothreitol, and 5 units of Taq DNA polymerase (Life Technologies, Inc.). The reaction mixtures were amplified for 30 cycles, with 1 min at 95°C for denaturation, 1.5 min at 53°C for annealing, and 2 min at 72°C for extension; one final extension step for 10 min at 72°C was also carried out. Amplification primers were designed as follows: sense oligonucleotide, 5'-GCAAGCTT-TCTAGATTTCTAGTTTCTGATACTTGGA-3' and antisense oligonucleotide 5'-GGGAGCTCAACATGGAGGGTTTTATAGTATAAATCA-3' from the mouse Nat3* gene. The PCR product was digested with HindIII and XhoI restriction enzymes.

Plasmid Construction. Mouse Nat1* (pCMV1) and Nat2* (pCMV2B6) were previously cloned by Martell et al. (1992). A 1,027-kb DNA fragment including the intronless coding region and adjacent segments of the 5' and 3' noncoding regions of Nat1* of the CD1 mouse strain was amplified by the polymerase chain reaction. Conditions for the amplification of Nat1* by PCR were as described above except for the final step of extension at 72°C, which was carried out for 120 min (Li and Guy, 1996). The sense (5'-GCGGAGCTCAACATGGAGGGTTTTATAGTATAAATCA-3') and antisense (5'-GGGAGCTCAACATGGAGGGTTTTATAGTATAAATCA-3') amplification primers included the SpeI and XhoI restriction sites, respectively. The pZeoSV vector and the Nat3*-PCR product were digested with SpeI and XhoI overnight at 37°C. The digested vector was dephosphorylated with 5 units of phosphatase for 1 hr at 37°C. The restriction enzyme-treated PCR fragment and the dephosphorylated vector were gel purified with Wizard purification system (Promega). Ligation reactions contained 25 ng of pZeoSV vector, 100 ng of PCR fragment, 10 μl ligation buffer, and 3 units of ligase (Promega). The reaction was allowed to proceed overnight at 16°C. Two μl of the ligation reaction was then transformed into TOP10 cells by electroporation. Selection of positive colonies was performed in the presence of 25 μg/ml Zeocin. The positive clones were analyzed for proper orientation and for the correct Nat3* nucleotide sequence (Sequencing DNA core, The University of Michigan, Ann Arbor, MI). The resulting construct was designated as pZeoSV3.

Expression of Recombinant Proteins. COS-1 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Hyclone, Logan, UT) and penicillin-streptomycin (Life Technologies, Inc.). Cells were transfected with lipofectamine reagent following the manufacturer's specifications (Life Technologies, Inc.). Briefly, COS-1 cells were grown in 100-mm tissue culture plates at 37°C in a 5% CO₂ incubator until they reached 70% confluency. For each transfection, 10 μl of plasmid DNA and 46 μl of lipofectamine reagent were combined and incubated at room temperature for 45 min. After the DNA-lipofectamine complexes were allowed to form, 2.4 ml of serum-free medium (OPTI-MEM, Life Technologies, Inc.) was added to the solution and overlaid onto rinsed cells. The transfection was allowed to continue for 5 hr at 37°C. Following incubation, the transfection mixture was removed and replaced with Dulbecco's modified Eagle's medium. Cell extracts were harvested 72 hr after the start of transfection and sonicated (0°C; 14 sec) in 400 μl of lysis buffer (20 mM Tris-HCl, pH 7.8, 1 mM EDTA, 1 mM dithiothreitol, 50 μM phenylmethylsulfon fluoride, 10 μM leupeptin) to disrupt cell membranes. Homogenates were centrifuged at 4°C at 14,000 rpm for 10 min in an Eppendorf centrifuge. The supernatant fractions were then centrifuged at 100,000 G for 1 hr and assayed for NAT activity and protein content as described below.

N-Acetylation Activity Determinations. Initial rates were determined with reaction mixtures containing cytosolic protein fraction in an amount determined to be proportional to protein content and to be linear with time in a 10-min reaction, substrate (0.05 mM 5-aminosalicylate, 0.1 mM p-amino benzoate, 0.1 mM 2-amino-2-fluorine, 0.5 mM sulfamethazine, or 0.1 mM p-aminobenzoic acid, 0.1 mM acetyl CoA) was used, an acetyl CoA regenerating system (4.5 mM acetyl-CD-caratine and 0.02 units of carinate acetyltransferase), 2 mM EDTA, 2 mM dithiothreitol, and 20 mM Tris-HCl buffer (pH 7.5 at 37°C) in a total volume of 100 μl (Andres et al., 1985). Substrate and acetyl CoA concentrations were chosen to conform with values used in the literature and were greater than their respective K₅ values (de León, 1996; Martell et al., 1992). The acetyl CoA regenerating system is used to maintain a nearly constant acetyl CoA concentration and to prevent the inhibitory effect of CoA. The 2-amino-2-fluorine reactions also contained 0.1% DMSO, a concentration previously shown not to alter Nat activity (Martell et al., 1992). Reactions were initiated with the addition of substrate, carried out for 10 min at 37°C, and were terminated with 10 μl of 15% perchloric acid or, in the case of p-aminobenzoic acid, 20 μl of glacial acetic acid or 100 μl of acetonitrile for 2-amino-2-fluorine reactions. After precipitation of the denatured protein, aliquots of the supernatant fraction were assayed for N-acetyl product formation by high pressure liquid chromatography with a reversed phase C₁₈ column, which was eluted at a flow rate of 1 ml/min. For p-aminobenzoate/5-acetyl-p-aminobenzoate, 5-aminosalicylate/5-acetyl-5-aminosalicylic acid, and sulfamethazine/5-acetyl-sulfamethazine, the solvent system was 0.1% trifluoroacetic acid/ methanol with detection at 266, 310, and 262 nm, respectively (de León, 1996). The analytical methods used are comparable with those previously described (Martell et al., 1992). For 2-amino-2-fluorine/2-acetamidofluorene, the solvent system was 20 mM potassium phosphate, pH 4.5, acetonitrile (66:34) with detection at 280 nm (Martell et al., 1992). For p-aminobenzoic acid/5-acetyl-p-aminobenzoic acid, the solvent system was 1% aqueous acetic acid/methanol with detection at 260 nm (Minchin, 1995). All compounds were quantitated by comparison of the integrated area of the elution peak with that of known amounts of the standard. NAT activities were normalized to the amount of protein as determined by the Bradford assay using bovine serum albumin standard (Bio-Rad). Isoniazid acetylator activity of the expressed NATs was assayed spectrophotometrically as described previously (Martell et al., 1992).

Results and Discussion

Amplification and Direct Sequencing of Nat3*. To investigate whether Nat3* shows genetic variation, the coding exon was amplified from liver genomic DNA from B6, A/J, and CD1 mouse strains. The sequence autoradiograms show that Nat3* is identical to the previously reported nucleotide sequence for Balb/c (Kelly and Sim, 1994). Fretland et al. (1997) also showed that Nat3* from C3H/HeJ and A/HeJ mouse strains is identical to Balb/c. To date, Nat3* has failed to show any coding region genetic variations among the mouse strains studied, which makes it similar to mouse Nat1* in that it may be classified as monomorphic.

Expression of Nat3* from CD1 Outbred Mouse Strain. To express mouse Nat1* in mammalian cells and evaluate the substrate selectivity of mouse Nat recombinant enzymes, the coding region and
5'- and 3'-untranslated regions were cloned into pZeoSV expression vector as specified in Materials and Methods. The sequence of the cloned Nat3* fragment was verified by direct sequencing and found to be identical to the genomic DNA nucleotide sequence except for a base change at position +12 (A → G), which does not alter the amino acid constitution of the protein.

Substrate Selectivity of Expressed Mouse Nats. Substrate selectivities were determined by arylamine N-acetyltransferase activity assay of cytosol from transiently transfected COS-1 cells with expression constructs of the intronless coding regions of Nat1*, Nat2**, previously cloned by Martell et al. (1992), and Nat3*. Table 1 shows the results of Nat activity determinations with sulfamethazine, p-aminobenzoic acid, isoniazid, p-aminoazobenzylglutamate, 2-aminofluorene, and 5-amino-saliclyic acid. Mock-transfected COS-1 cells showed background activity (less than 0.04 nmol/min/mg protein) with these substrates, which was subtracted from the sample determinations. Nat1 showed a low specific acetylatng activity with sulfamethazine (2 × background) in contrast to previous studies that failed to detect any activity for this substrate with recombinant Nat1 (Martell et al., 1992) and Nat3 (Fretland et al., 1997). In agreement with previous studies, isoniazid was found to be a Nat1-specific substrate (table 1). p-Aminobenzoic acid is a selective substrate for recombinant mouse Nat2, which showed 20-fold higher acetylation activity toward this substrate than did Nat1. 2-Aminofluorene and 5-aminosalicylic acid showed a higher activity with Nat2 than Nat1 (5.4- and 2.4-fold, respectively) but were both actively acetylated by both Nats. Nat3 showed only a slight activity with 5-aminosalicylic acid and no detectable activity with any of the other tested substrates. The Nat3 activity was 8.5- and 20-fold lower than Nat1 and Nat2, respectively. p-Aminobenzoylglutamate (pABG) has only recently been explored as a potential NAT endogenous substrate. pABG is a metabolite of folic acid that is N-acetylated to form N-acetyl-p-aminobenzoylglutamate (N-Ac-pABG). Minchin (1995) investigated the acetylation of pABG by U937 cells and recombinant human NATs expressed in COS cells. He showed that pABG is a specific substrate for human NAT1 and proposed pABG to be a potential human NAT1 endogenous substrate, the first of such to be identified. We examined the selectivity of pABG with recombinant mouse Nats and found it to be a specific substrate for mouse Nat2.

Although saturating substrate concentrations were employed in this study, determination of kinetic constants would be most informative. Nevertheless, in addition to supplying useful markers for the various mouse Nat enzymes, the substrate selectivity profile observed further supports the functional analogy between mouse Nat2 and human NAT1: substrates that are specific or selective for human NAT1 are selective for mouse Nat2 and vice versa, a hypothesis also supported by the finding that mouse Nat2 exhibits higher percentage identity of deduced amino acid sequence with human NAT1 (80%) than with human NAT2 (74%) (Vatsis et al., 1995).

This report is the first of mouse Nat3 being expressed in mammalian cells. Previously, the substrate selectivity of mouse Nat3 expressed in Escherichia coli has been complicated by formation of insoluble aggregates (protein inclusions) and production of very little active enzyme (Kelly and Sim, 1994). In another report (Fretland et al., 1997), mouse Nat3 was also expressed in E. coli, and the O-acetylation and N,O-acetyltransferase activities were measured. O-Acetylation was not detectable, and N,O-acetyltransferase was 200- and 600-fold less than Nat1 and Nat2, respectively. In the three studies reported (Fretland et al., 1997; Kelly and Sim, 1994; this paper), the actual level of Nat3 expression has not been determined. Neither the anti-human NAT1 nor anti-human NAT2 used by Fretland et al. (1997) recognized bacterially expressed mouse Nat3. Similarly, our anti-NAT antibody did not allow us to measure specific Nat protein expression.

The information reported in this paper provides further understanding of mouse Nats as a model of the human enzymes and their contribution in the metabolism of therapeutic agents, carcinogenic compounds, and endogenous substrates such as folic acid metabolites. We report that the metabolism of compounds such as p-aminobenzoic acid and pABG is primarily accomplished by mouse Nat2, which corresponds to human NAT1. On the other hand, the study of substrates such as sulfamethazine and isoniazid indicate that they are primarily substrates for Nat1, which corresponds to human NAT2. Thus, it is possible that the low activity reported for Nat3 may be due to low expression; however, the relative activities of Nat3 toward various substrates should not be greatly altered by low expression.

The physiological significance of mouse Nat3 remains unresolved. The failure to detect significant acetyltransferase activity with this isozyme may be due to poor expression in both prokaryotic and eukaryotic systems, failure to use an appropriate Nat3 substrate, intrinsic instability of the enzyme, or inappropriate identification of Nat3 as an active acetyltransferase.

Acknowledgments. We thank Dr. R. Minchin and Dr. M. Montrose for gifts of materials.

Department of Pharmacology
(L.E.-R., G.N.L., W.W.W.)
The University of Michigan
Medical School

References


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**TABLE 1**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Nat1</th>
<th>Nat2</th>
<th>Nat3</th>
</tr>
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<tbody>
<tr>
<td>Isoniazid*</td>
<td>35.04 ± 1.42</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Sulfamethazine</td>
<td>0.86 ± 0.06</td>
<td>0.08 ± 0.01</td>
<td>ND</td>
</tr>
<tr>
<td>PABA</td>
<td>0.44 ± 0.22</td>
<td>9.14 ± 0.31</td>
<td>ND</td>
</tr>
<tr>
<td>pABG</td>
<td>ND</td>
<td>15.30 ± 0.80</td>
<td>ND</td>
</tr>
<tr>
<td>2-AF</td>
<td>2.03 ± 0.19</td>
<td>10.88 ± 1.17</td>
<td>ND</td>
</tr>
<tr>
<td>5-ASA</td>
<td>7.19 ± 0.49</td>
<td>17.56 ± 0.39</td>
<td>0.85 ± 0.28</td>
</tr>
</tbody>
</table>

ND, less than 0.04 nmol/min/mg of protein for sulfamethazine, p-aminobenzoylglutamate (PABA), pABG, 2-aminofluorene (2-AF), and 5-amino-saliclyic acid (5-ASA); less than 0.1 nmol/min/mg of protein for INH.

* Assay differed substantially from all other substrates (see text).

† Values represent averages of specific activity (nmol/min total protein) ± standard errors (N = 3). Background values for activity (<0.04 nmol/min/mg of protein) have been subtracted.


