A Second Mammalian N-Myristoyltransferase*

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N-terminal myristoylation is a cotranslational lipid modification common to many signaling proteins that often serves an integral role in the targeting and/or function of these proteins. Myristoylation is catalyzed by an enzyme activity, N-myristoyltransferase (NMT), which transfers myristic acid from myristoyl coenzyme A to the amino group of a protein's N-terminal glycine residue. While a single human NMT cDNA has been isolated and characterized (hNMT-1), biochemical evidence has indicated the presence of several distinct NMTs in vivo, often varying in either apparent molecular weight and/or subcellular distribution. We now report the cloning and characterization of a second, genetically distinct human NMT (hNMT-2), as well as the isolation of the respective mouse NMT homologue for each human enzyme. The mouse and human versions of each NMT are highly homologous, displaying greater than 95% amino acid sequence identity. Comparisons between the NMT-1 and NMT-2 proteins revealed reduced levels of sequence identity (76–77%), indicating that NMT-1 and NMT-2 comprise two distinct families of N-myristoyltransferases. Transient transfection of either the hNMT-1 or hNMT-2 cDNA into COS-7 cells resulted in the expression of high levels of NMT enzyme activity. Both hNMT-1 and hNMT-2 were found to myristoylate several commonly studied peptide substrates with similar, but distinguishable, relative selectivities. Western analysis revealed that while hNMT-2 appeared with similar, but distinguishable, relative selectivities.

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‡ The abbreviations used are: NMT, N-myristoyltransferase; hNMT, human N-myristoyltransferase; mNMT, mouse N-myristoyltransferase; EST, expressed sequence tag; GST, glutathione S-transferase; PKA, cAMP-dependent protein kinase; PCR, polymerase chain reaction; kb, kilobase pair(s).

The cotranslational modification of proteins with myristic acid serves to regulate both protein function and localization (1, 2). Most myristoylated proteins are acylated through an amide linkage to their N-terminal glycine residues, a reaction catalyzed by the enzyme, N-myristoyltransferase (NMT) (EC 2.3.1.197) (3–5). Myristoylation has proven essential to the biological activity of many mammalian, viral, and fungal proteins. In particular, the transformation potential of the protein tyrosine kinase, p60<sup>src</sup>, is entirely dependent on myristoylation, as nonmyristoylated forms of p60<sup>src</sup> fail to bind cellular membranes and are transformation defective (6). Similarly, nonmyristoylated forms of endothelial nitric-oxide synthase are not properly localized to the Golgi apparatus and plasma-membrane caveolae, resulting in marked reductions in stimulated nitric oxide production (7, 8). The dependence of viral infectivity on myristoylation is exemplified by the observation that inhibiting the myristoylation of the human immunodeficiency virus type I GAG precursor protein promotes the production of noninfectious viral particles (9).

Genetic studies have shown that the NMT gene is essential for the viability of the yeast, <i>Saccharomyces cerevisiae</i> (10), and the pathogenic fungi, <i>Candida albicans</i> (11) and <i>Cryptococcus neoformans</i> (12). Accordingly, inhibitors of <i>C. albicans</i> NMT have proven to be potent antifungal agents (13). In mammalian systems, NMT activity has been shown to increase in colorectal tumors (14, 15), leading to the proposal that NMT could serve as a target for anticancer therapies (16). In this regard, one speculated mechanism for the antitumor activity of the natural product fumagillin is through indirectly preventing protein myristoylation (17). Fumagillin has been shown to inhibit the methionine aminopeptidase, MetAP-2, an enzyme that cleaves the N-terminal methionine from newly synthesized proteins (17), a process required for the exposure of N-terminal glycine residues of NMT protein substrates.

A single human NMT cDNA has been isolated and characterized (18, 19), and subsequent failures to identify homologous human cDNAs has led some to speculate that NMT activity in <i>vivo</i> is likely derived from a single gene (20). However, biochemical studies have repeatedly indicated the presence of multiple distinct protein forms of NMT in <i>vivo</i>, often varying in either molecular size and/or subcellular distribution (2, 21–23). Whether all of these NMT forms are derived from a single gene or from multiple NMT genes has remained unclear (2). We now report the isolation and characterization of a second distinct NMT cDNA from a human liver library, as well as the cloning of the respective mouse homologue for each of the two human NMTs. For the sake of clarity, we will hereafter refer to the originally characterized human NMT as hNMT-1 and the human NMT described in the present study as hNMT-2.

**Experimental Procedures**

Cloning of Human and Mouse NMT cDNAs—PCR primers based on the sequence of expressed sequence tag (EST) AA036845 were designed for the amplification of a 550-base pair portion of the hNMT-2 cDNA from a human liver 5’Stretch Plus cDNA library (CLONTECH): Primer 1, 5’-GGGAATTCAACATCCACACAGAGACGCCC-3’; Primer 2, 5’-GGGATCTCTGTAACACTCTGAGACGCC-3’. This amplified DNA was used as a probe to screen human and mouse liver 5’Stretch Plus cDNA libraries according to the manufacturer’s guidelines. Four positive human libraries according to the manufacturer's guidelines. Four positive human libraries according to the manufacturer's guidelines. Four positive human libraries according to the manufacturer's guidelines. Four positive human libraries according to the manufacturer's guidelines. Four positive human libraries according to the manufacturer's guidelines. Four positive human libraries according to the manufacturer's guidelines. Four positive human libraries according to the manufacturer's guidelines. Four positive human libraries according to the manufacturer's guidelines. Four positive human libraries according to the manufacturer's guidelines. Four positive human libraries according to the manufacturer's guidelines. Four positive human libraries according to the manufacturer's guidelines. Four positive human libraries according to the manufacturer's guidelines. Four positive human libraries according to the manufacturer's guidelines. Four positive human libraries according to the manufacturer's guidelines. Four positive...
human clones and six positive mouse clones were isolated from screenings of 3.2 × 10⁶ and 9.6 × 10⁵ plaques, respectively. A 2.85-kb human clone and a 1.9-kb mouse clone contained the presumed complete coding sequence for hNMT-2 and mNMT-2, respectively. The hNMT-1 DNA was isolated by PCR with primers based on the reported GenBank™ sequence (accession number AF020500): Primer 1, 5′-GAAGCTTCTGCAAGAGGAGATG-3′, Primer 2, 5′-GAATTCAGTTCT-GCTCCCTTTGCC-3′. This hNMT-1 cDNA was then used as a probe to screen both human brain and mouse liver cDNA libraries (CLONTECH; 3.2 × 10⁶ plaques per screening). Six positive human clones and eight positive mouse clones were identified. A 4.4-kb human clone and 1.6-kb mouse clone encoded the presumed complete coding sequence for hNMT-1 and mNMT-1, respectively. All reported cDNAs were cloned into the eukaryotic expression vector, pcDNA3, and transiently transfected into COS-7 cells as described previously (24), with the exception that 1.25 × 10⁵ plaques per screening. Six positive human clones and eight positive mouse clones were identified. A 4.4-kb human clone and 1.6-kb mouse clone encoded the presumed complete coding sequence for hNMT-1 and mNMT-1, respectively. All reported cDNAs were cloned into pBluescript II SK(+) and sequenced completely in both directions. NMT protein sequence comparisons were conducted using the DNASTAR/Clustal program (Clustal method), and percentage sequence identities were calculated from sequence alignments after excluding the identical gaps. For Northern analysis, cDNA probes specific for the hNMT-1 and hNMT-2 genes were generated from the 5′ most 400 base pairs and 99 base pairs, respectively. The hNMT-1 cDNA was then used as a probe to screen both human brain and mouse liver cDNA libraries (CLONTECH; 3.2 × 10⁶ plaques per screening). Six positive human clones and eight positive mouse clones were isolated from screenings described previously (24), with the exception that 1.25 μg of the hNMT-1 cDNA was used per transfection to reduce the amount of hNMT-1 expression to levels comparable with hNMT-2 expression (as judged by NMT enzyme activities). Transfected cells were harvested by trypsinization, washed twice with Hepes buffer (12.5 mM Hepes, pH 8.0, 1 mM EDTA, 100 mM NaCl), and Dounce-homogenized in Hepes buffer containing 1× complete protease inhibitors (Boehringer Mannheim) on ice. The homogenized cell extracts were then sonicated briefly (5 s), assayed for protein content (D₅₀ protein assay kit, Bio-Rad), and used for enzyme assays, Western blotting, and cell fractionation experiments (25).

Generation of Anti-NMT Antibodies—The hNMT-1 cDNA was cloned into the pGEX-4T-3 vector (Amersham Pharmacia Biotech) for generation of a GST-hNMT-1 fusion protein (residues 89–496 of hNMT-1) according to manufacturer’s guidelines. Rabbit polyclonal antibodies were generated against the GST-hNMT-1 fusion protein (residues 89–496) and peptide substrate (200 μM) in a reaction buffer of 30 mM Tris-HCl, pH 7.5 with 0.5 mM EDTA, 0.5 mM EGTA, 1.0% (v/v) Triton X-100, and 4.5 mM β-mercaptoethanol (total reaction volume of 50 μl; reaction buffer from King and Sharma (27)). The reaction was allowed to proceed for 10 min at 25 °C, then quenched with 50 μl of methanol followed by 5 μl of 100% trichloroacetic acid, placed on ice for 10 min, and spun at 10,000 × g for 5 min. Aliquots (25 μl) of the supernatant were analyzed by reverse-phase high pressure liquid chromatography. A myristoylated GNNAAAAR peptide was synthesized as described by Towler and Glaser (26) and used as a standard to define the elution times for myristoylated peptide products. Column fractions (1 ml) were collected and counted by scintillation counting. In all cases, control reactions without peptide were also analyzed and subtracted from reactions with peptide to provide the myristoylation rates reported in Table I. Each reaction was run in triplicate with data reported as the average ± S.D. of these values. Initial rates were determined from reactions in which less than 20% myristoylated product was formed. Peptide substrates were designed based on previous work of Boutin and colleagues (28).

RESULTS AND DISCUSSION

Cloning of Human and Mouse NMTs—Intrigued by an apparent disparity between the available biochemical and genetic

**FIG. 1.** Comparison of the deduced amino acid sequences from human NMT-1 and NMT-2 cDNAs. Shared sequence identities between NMT-1 and NMT-2 are shaded.

**FIG. 2.** Comparisons of the deduced amino acid sequences from human and mouse NMT cDNAs. A, comparison of the human and mouse NMT-1 proteins (hNMT-1 and mNMT-1, respectively); B, comparison of the human and mouse NMT-2 proteins (hNMT-2 and mNMT-2, respectively). Shared sequence identities between the human and mouse NMTs are shaded.
TABLE I
N-Myristoyltransferase activities of hNMT-1- and hNMT-2-transfected COS-7 cells

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Mock-transfected myristoylation rate</th>
<th>hNMT-1-transfected myristoylation rate</th>
<th>hNMT-1 myristoylation rate</th>
<th>hNMT-2-transfected myristoylation rate</th>
<th>hNMT-2 myristoylation rate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pmol · min⁻¹ · mg⁻¹</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>Src peptide (GSSKS KPDP)</td>
<td>6.4 ± 0.2</td>
<td>34.7 ± 1.4</td>
<td>100</td>
<td>38.5 ± 1.6</td>
<td>100</td>
</tr>
<tr>
<td>PKA peptide (GNAAAR)</td>
<td>1.9 ± 0.1</td>
<td>21.9 ± 0.7</td>
<td>70</td>
<td>17.9 ± 0.3</td>
<td>50</td>
</tr>
<tr>
<td>c-Abl peptide (GQQPGKVL)</td>
<td>1.0 ± 0.3</td>
<td>13.2 ± 0.7</td>
<td>43</td>
<td>9.5 ± 1.5</td>
<td>27</td>
</tr>
<tr>
<td>Tumor necrosis factor peptide (REALPKRT GOQPQSK)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
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N-Myristoyltransferase activity measured in transfected COS-7 cell extracts with various potential peptide substrates. Mock-transfected COS-7 cells were transfected with the pcDNA3 vector alone. For hNMT-1 and hNMT-2, myristoylation rates with the Src peptide were considered to be 100% of hNMT activity, to which other peptide myristoylation rates were compared. Absolute myristoylation rates were calculated after subtraction of control values from reactions run in the absence of peptide, with data reported as the average of three trials ± S.D. Percentile hNMT myristoylation rates were calculated after subtraction of mock transfection myristoylation rates. ND = no detectable activity.

...data on N-myristoyltransferase enzymes, we searched the EST database for homologues to a previously characterized human NMT cDNA (hNMT-1). Identified ESTs separated into two distinct categories, either being identical to the hNMT-1 cDNA or approximately 70–80% identical to the hNMT-1 cDNA (e.g., EST AA203325, AA036845, AA036785, AA364769). Moreover, the collection of homologous, but distinct, ESTs appeared to be derived from a common cDNA, leading us to conclude that a second as of yet uncharacterized NMT existed in humans. Oligonucleotide primers based on the cDNA sequence of EST AA036845 were used in the PCR to amplify a 550-base pair fragment of the novel NMT cDNA from a human liver cDNA library. This PCR product was subsequently used to isolate a 2.85-kb cDNA that encoded a putative full-length NMT (hNMT-2). Although no stop codon was identified upstream of the first encoded methionine residue, the sequence surrounding this ATG matched well with the Kozak sequence for predicted eukaryotic translation initiation sites (29). Additionally, this putative translation initiation site was shared by all four mammalian NMT cDNAs and coded for the only conserved in-frame methionine residue upstream of the previously defined NMT ribosomal targeting domain (19) (see below). Comparisons between the predicted protein sequences for hNMT-1 and hNMT-2 demonstrated that the two proteins shared 77% amino acid identity (Fig. 1). Sequence divergence was most prevalent in the N-terminal domains of the NMTs, a region of hNMT-1 dispensable for catalytic activity, but implicated in protein targeting (19, 30). In this regard, an intriguing conserved sequence was noted in this otherwise dissimilar region of the NMTs, as amino acids 54–68 of hNMT-1, GAKKKKKQKKKEK, matched nearly identically amino acids 44–58 of hNMT-2, GAKKKKKQKKKEK. Similar stretches of positively charged residues have been identified in other proteins involved in the cotranslational processing of proteins, including N-methionylaminopeptidase (31), leading to the proposal that these sequence elements serve to target such enzymes to the ribosome (19).

To gain a better understanding of the potential significance of the sequence divergence between hNMT-1 and hNMT-2, their respective mouse homologues, mNMT-1 and mNMT-2, were isolated and characterized. Interestingly, the mouse and human NMTs segregated neatly into two distinct pairs of enzymes based on their homologies in primary structure (Fig. 2), with mNMT-1 and hNMT-1 sharing 97% amino acid sequence identity and hNMT-2 and mNMT-2 sharing 96% amino acid sequence identity. One notable difference between mNMT-2 and hNMT-2 was the presence in the former of an inserted stretch of 31 amino acids in the N-terminal portion of the protein (amino acids 84–114). Further examination of the mNMT-2 cDNA failed to identify consensus intron splice sites within this inserted sequence, indicating that amino acids 84–114 were a legitimate part of the mNMT-2 protein. Additionally, five independent mNMT-2 cDNA clones were isolated and one EST (W62224) identified that covered this region of the mNMT-2 sequence and each of these clones possessed this insert. A comparison between the mNMT-1 and mNMT-2 proteins revealed a reduced level of sequence identity of 76%, reminiscent of the homology found between hNMT-1 and hNMT-2. Thus, most of the distinguishing features between the primary structures of NMT-1 and NMT-2 have been conserved from mouse to human. Northern analysis demonstrated that most human and mouse tissues concurrently express both NMTs, indicating that a functional difference between the two enzymes is not likely rooted in restricted tissue distribution profiles (data not shown).

Expression of hNMTs in COS-7 Cells—To compare the hNMT-1 and hNMT-2 proteins further, each NMT cDNA was cloned into the eukaryotic expression vector pcDNA3 and transiently transfected into COS-7 cells. COS-7 cells expressing either hNMT-1 or hNMT-2 showed significantly higher levels of NMT activity than mock-transfected cells (Table I). Several peptides were tested as substrates for each NMT to gain a preliminary understanding of the relative substrate specificities of each enzyme. Both hNMT-1 and hNMT-2 myristoylated peptide substrates corresponding to the N termini of cAMP-dependent protein kinase (PKA), Src kinase, and Abl kinase (Table I). Analysis of the initial reaction rates revealed that the enzymes shared similar substrate specificities, with the notable exception that hNMT-2 showed a greater relative prefer-
ence for the Src peptide substrate. Neither NMT myristoylated either 1) a control peptide in which the N-terminal glycine from the PKA peptide was removed or 2) an internal peptide from the tumor necrosis factor α precursor protein, EELPKKTGG-PQGSR, which is myristoylated on lysine residues (32).

Affinity-purified antibodies generated against a GST fusion protein of hNMT-1 were used to characterize by Western blotting the expression of each NMT in COS-7 cells (Fig. 3). hNMT-1 and hNMT-2 appeared as primarily single protein bands of 68 and 65 kDa in size, respectively (Fig. 3, lanes 4 and 7). While these apparent sizes for hNMT-1 and hNMT-2 were larger than their predicted molecular masses (about 57 kDa for each enzyme), previous work has indicated that NMTs migrate anomalously large on SDS-polyacrylamide gel electrophoresis (19). Interestingly, three additional isoforms of hNMT-1 were also identified in transfected COS-7 extracts (apparent sizes of 49, 55, and 56 kDa; lane 4), while no additional forms of hNMT-2 were observed (lane 7). The multiple hNMT-1 proteins were likely the result of in vivo processing events, as an equivalent hNMT-1 protein profile was identified in transfected COS-7 cells harvested directly into standard SDS gel loading buffer (data not shown). Mock-transfected COS-7 cells showed weakly immunoreactive 64- and 67-kDa proteins that may represent endogenous NMTs (lanes 1–3). Separation of COS-7 extracts into cytoplasmic and membrane fractions revealed that hNMT-2 and the 68-kDa isoform of hNMT-1 appeared significantly in both the cytoplasmic and membrane fractions (lanes 5, 6, 8, and 9), while the 49-, 55-, and 56-kDa isoforms of hNMT-1 were predominately cytoplasmic (lane 5). The majority of each NMT enzyme activity was found in the cytoplasm (85 and 75% for hNMT-1 and hNMT-2, respectively). However, such relative enzyme activity comparisons should be interpreted with caution, given that membrane associated inhibitory factors have been shown to reduce the apparent activity of membranous NMTs (33). Finally, considering that hNMT-1 has recently been shown to target to ribosomes (19), the membranous NMTs identified here may in fact reflect association of these proteins with ribosomes that are themselves bound to membranes.

Conclusions—The isolation and characterization of a second family of mammalian N-myristoyltransferases has profound implications on both the in vivo regulation and pharmaceutical targeting of protein myristoylation. The strict conservation of primary structure identified within both NMT-1 and NMT-2 families suggests unique functions for each enzyme. In mouse and human tissues examined, while a preliminary analysis of these proteins with ribosomes that are themselves bound to membranes.

part that each NMT plays in supporting tumorigenesis and/or viral infectivity. An exciting possibility arises for selective pharmaceutical intervention if certain tumors or viruses require the activity of primarily one type of NMT. In this regard, NMT activity and protein levels have been found to increase several fold in human colorectal tumors (14, 15). Through the generation of NMT-1- and NMT-2-specific antibodies, the identification of the up-regulated NMT(s) in these tumors should be possible. Such NMT-1- and NMT-2-specific reagents in union with further examinations into the catalytic properties and cellular regulation of each enzyme should provide significant insights into the in vivo role of protein myristoylation in both normal and disease states.

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REFERENCES