RESEARCH COMMUNICATION

Characterization of human and rat brain myristoyl-CoA:protein N-myristoyltransferase: evidence for an alternative splice variant of the enzyme

R. A. Jeffrey McILHINNEY^{*1}, Kate YOUNG^{*}, Mark EGERTON[†], Roger CAMBLE[†], Ann WHITE[†] and Mikhail SOLOVIEV^{*}

^{*}Medical Research Council Anatomical Neuropharmacology Unit, Mansfield Road, Oxford OX1 3TH, U.K., and [†]Research Department, Zeneca Pharmaceuticals, Mereside, Alderley Park, Macclesfield, Cheshire SK10 4TG, U.K.

Using 5'-rapid amplification of cDNA ends, we have identified an extended 5'-end of mRNA coding for human myristoyl-CoA:protein N-myristoyltransferase (NMT). PCR using primers based on this new 5'-sequence and reverse primers within the currently accepted coding sequence of the enzyme resulted in the identification of a novel splice variant of NMT. *In vitro* translation of these cDNAs resulted in the production of proteins with

apparent molecular masses of 63 kDa and 48 kDa. Immunoprecipitation of NMT from human cell lines and immunoblotting of a range of rat tissues has identified proteins with molecular masses corresponding to those derived from these cDNAs, and provided evidence that their relative abundance differs among tissues. Our results provide evidence that this enzyme exists in different forms resulting from alternative splicing of the mRNA.

INTRODUCTION

A wide range of cellular proteins are now known to be post-translationally modified by the covalent attachment of myristic acid to an N-terminal glycine. The enzyme responsible for the attachment of myristic acid to proteins is myristoyl-CoA:protein N-myristoyltransferase (NMT), and protein myristoylation is now recognized to be both a widespread and functionally important modification of proteins [1–3]. Several viruses, including hepatitis B [4], poliovirus [5–7] and HIV [8,9], have been shown to require the myristoylation of coat or regulatory proteins for viral assembly and/or replication. Indeed, both heteroatom-substituted analogues of myristic acid [10] and inhibitors of NMT have been reported to inhibit the replication of a number of viruses, including HIV [11–13].

Fungi contain a single gene coding for NMT and mutations that render the enzyme inactive are lethal, whereas mutations that reduce the catalytic efficiency of the enzyme cause myristic acid auxotrophy and temperature-sensitive growth [14–17]. In addition, heteroatom-substituted myristic acid analogues, which are incorporated into fungal myristoylated proteins and alter their properties, have been shown to be fungicidal for *Cryptococcus neoformans* [18]. Since both viral and fungal growth can be inhibited by perturbation of myristoylation, NMT seems to be a suitable target for the development of therapeutic agents directed against these pathogens.

The original cloned cDNA for NMT had two potential in-frame start sites as residues 28 and 211 respectively [19], the second of which was proposed to be the site for initiation of protein synthesis. Several laboratories have since expressed the protein produced using the second start site in *Escherichia coli* and obtained active enzyme [19–21]. However, the protein produced from the initiation codon ATG at position 211 has a predicted molecular mass of 48 kDa and, though active, is significantly smaller than the purified bovine brain enzyme. Furthermore, immunocytochemical studies of human NMT in

HeLa cells, using both polyclonal and anti-peptide antisera, showed that the antisera recognized a protein of 63 kDa, which was larger than a bacterially expressed histidine-tagged NMT based on a protein initiated from ATG at position 211 [22]. Consequently, it has been suggested that the alternative start site at position 28 in the cDNA may be used for synthesis of mammalian NMT [22,23]. A recent report has suggested that this is the case and that the additional sequence is responsible for the polyribosomal targeting of NMT [23].

Clearly, because of the interest in the enzyme as a potential therapeutic target, it is important to know whether either of these potential initiation sites in the cloned cDNA is used *in vivo* for synthesis of the enzyme, or whether there are additional sites responsible for initiation of protein translation. We report here both the identification of a human cDNA, which contains an extended 5'-mRNA sequence, and an alternatively spliced mRNA for NMT. The former encodes a protein of 63 kDa, whereas the latter would encode a protein of 48 kDa, and proteins with similar molecular masses can be immunoprecipitated from both cell lines and subsequently identified in rat tissues.

MATERIALS AND METHODS

Materials

Protein A-Sepharose was obtained from Sigma. Tran ³⁵S label (> 1000 Ci/mmol) was obtained from ICN Biomedicals (Thame, Oxon, U.K.). Nitrocellulose sheets were obtained from Schleicher and Schuell (Dassel, Germany) and Immobilon PVDF membranes were from Millipore (Bedford, MA, U.S.A.). Tissue culture materials were obtained from Gibco Life Technologies (Paisley, U.K.). All other reagents were from Merck, and were of the highest quality obtainable. The TNT quick coupled transcription/translation system was purchased from Promega (Southampton, Hants., U.K.) Pre-stained molecular-

Abbreviations used: NMT, myristoyl-CoA:protein N-myristoyltransferase; RACE, 5'-rapid amplification of cDNA ends; poly(A)⁺, polyadenylated.

¹ To whom correspondence should be addressed (e-mail jeff.mcilhinney@pharm.ox.ac.uk).

mass markers were obtained from Novex (San Diego, CA, U.S.A.).

Identification of the alternatively spliced NMT mRNA

Polyadenylated [poly(A)⁺] RNA was isolated from human embryonic kidney 293 cells, from HeLa cells and from human lymphocytes using the Pharmacia Quick Prep kit (Milton Keynes, U.K.). For cDNA synthesis, each of the mRNAs was incubated in the first-strand reverse transcriptase buffer containing 2 µg/ml of the random hexamer primer, 10 mM dithiothreitol, 1 mM each of dATP, dTTP, dCTP and dGTP and 10 units/µl of the SuperScript II Moloney murine leukaemia virus reverse transcriptase (Gibco BRL, Cergy-Pontoise, France). Incubation was for 10 min at 22 °C, followed by 1 h at 42 °C and a further at 55 °C. The first-strand reaction mix was used for PCR amplification without further purification. For the 5'-rapid amplification of cDNA ends (RACE)-PCR amplification, the cDNAs were precipitated with ethanol from the first-strand reaction mix after hydrolysis, with 0.4 M NaOH of the remaining mRNA. The cDNA samples were incubated with 3 pmol/µl of the AD adaptor oligonucleotide (5' - GAAGGTACCAAGCTTGGGTCTCCCTATAGTGAGTCGTATTA-3') and 2 units/µl of the T4 RNA ligase (Gibco BRL) in 2 × T4 DNA ligase buffer (Boehringer Mannheim, Mannheim, Germany) for 1 h at 4 °C followed by 4 h at 37 °C. The tailing reaction mix was used for RACE-PCR amplification without further purification. Primers used for the first round of RACE-PCRs were AD-F_{out} (5'-TAATACGACTCACTATAGGG-3') and NMT-R_{out} (5'-TGTAGGGCTCTGGCGGATATTGTCTTGTC-3'). The second round of RACE-PCRs employed nested primers AD-F_{in} (5'-GGGAGACCCAAGCTTGGTACCTTC-3') and NMT-R_{in} (5'-GGACGGGCTGCGTATCCCAGAACT-3'), and 1 µl of the corresponding first-round RACE-PCR mix. All amplifications were carried out using proof reading *Pfu* DNA polymerase. Amplification conditions were 1 cycle of 1 min at 96 °C, 1 min at 65 °C, 5 min at 72 °C, and 30 cycles of 1 min at 96 °C, 1 min at 65 °C and 2 min at 72 °C, followed by 10 min at 72 °C. The PCR products were extracted from 2% (w/v) agarose gels and subcloned into the pcDNA3 vector (Invitrogen, Leek, The Netherlands).

The existence of alternatively spliced forms of the 5'-end of NMT mRNA was shown using two pairs of nested primers to amplify that region. The downstream primers were NMT-R_{out} and NMT-R_{in} (as above), and the upstream primers were NMT-F_{out} (5'-GGAGGTACCCTGCTCTCGCAACTCAAGATG-3') and NMT-F_{in} (5'-CCTCCGGTACCGCAGATGATGGAA-GGG-3'). First-strand cDNA synthesis reaction mixes were used as the templates for amplification of the two alternatively spliced forms of NMT mRNA. PCR conditions, and subsequent sub-cloning of the amplified products, were as found for RACE-PCRs (see above).

To generate full-length NMT clones, the new 5'-mRNA ends of both the long and the short forms of the NMT were generated using the NMT-F_{out} and NMT-R_{out} primers, *Pfu* DNA polymerase and first-strand cDNA reaction mix as a template. The amplification conditions were as described above for RACE-PCR. Human NMT cDNA containing ATG at position 28 of the original NMT cDNA sequence [19] was obtained from Zeneca Pharmaceuticals (Alderley Park, Macclesfield, Cheshire, U.K.) and cloned into pcDNA3 to give the plasmid pNMT-M. *KpnI*/*Bam*HI fragments of each of the long and the short forms of NMT cDNA were incorporated into the *KpnI*/*Bam*HI-digested pNMT-M (the *KpnI* site is in the vector polylinker sequence and the *Bam*HI site is contained within the

cDNA for human NMT) to produce plasmids pNMT-L and pNMT-S [encoding the long (NMT_L) and short (NMT_S) forms of the enzyme accordingly; see Figure 2].

Production of antisera

Human NMT cDNA, a full-length NMT_S (see Figure 2) was subcloned into the bacterial expression vector pTB361, which was derived from pBR322 by the introduction of the T7 promoter [24], to produce the plasmid pTBNMT, and this was introduced into the *E. coli* strain BL21(DE3). Induction of protein expression with isopropyl β-D-thiogalactopyranoside resulted in the production of a 48 kDa protein, which was fully enzymically active. Inclusion bodies (100 µg of protein) containing NMT_S from induced cultures of pTBNMT were used in Freund's complete adjuvant to immunize rabbits by subcutaneous injection. Continuing immunization of the rabbits and affinity purification using nitrocellulose-absorbed NMT were performed as described previously [22]. This antibody reacts with all forms of NMT.

Cell culture

Culture, metabolic labelling of HeLa and HEK 293 cells with 100 µCi/ml trans ³⁵S label, immunoprecipitation of NMT and cell cytosol preparation were performed as described previously [22,25].

SDS/PAGE and immunoblotting

Analysis of proteins on SDS/polyacrylamide gels and transfer of proteins to PVDF membranes, and their subsequent detection using alkaline-phosphatase-conjugated second antibodies, were carried out as described previously [26]. In all of these experiments, the primary antibody was affinity-purified and used at a concentration of 100 ng/ml.

In vitro translation of NMT

The Promega TNT rabbit reticulocyte-lysate-coupled translation system was used throughout using T7 polymerase to produce the RNA from subunit cDNAs cloned into pcDNA1/Amp. The total amount of DNA in each reaction was 0.5 µg. The final volume of each reaction was 25 µl containing 2.5 µCi Tran ³⁵S-methionine and 20 µl of reticulocyte lysate. Incubations were performed at 30 °C for 90 min.

Tissue preparation

All rat tissues were dissected rapidly, placed on an ice-cooled platform and minced using a scalpel. Samples (0.15 g) from each tissue were frozen immediately in liquid-N₂ before being solubilized in 500 µl of SDS sample buffer by heating to 100 °C for 5 min. The resulting solution was centrifuged (12 300 g for 15 min) at 4 °C and aliquots were stored at -70 °C. Protein determinations were carried out using the Bio-Rad dye-binding assay and BSA as a standard.

RESULTS AND DISCUSSION

We have used human poly(A)⁺ RNA from the human cell lines HEK 293 and HeLa, as well as from human lymphocytes, in RACE-PCR amplification of human NMT. The 3'-NMT-specific primers were based on the known sequence of human NMT,

Figure 1 Cloning of the two alternatively spliced forms of human NMT

(A) 5'-RACE-PCR amplification of human NMT. The only specific product (major band of ≈ 420 bp) encodes the full-length 5'-end of human NMT-L in all of the mRNAs tested. The faint band seen in lane 3 is a non-specific product, as determined by DNA sequencing. (B) Reverse transcriptase-PCR amplification of NMT-L (476 bp, major product) and of NMT-S (367 bp, minor product). (C) Reamplification of the 367-bp gel-purified DNA (see 1B) using nested primers reveals the presence of NMT-S in all mRNAs tested. In all panels: HEK cells (1), HeLa cells (2) and human lymphocytes (3). Molecular size markers (Gibco BRL) are shown in bp.

whereas the 5'-primers were adaptor-specific. Two rounds of the RACE-PCR resulted in the amplification of a major product, which was identical in size (approx. 420 bp) for each of the three template cDNAs (Figure 1A). Sequencing of the amplified cDNA revealed the adaptor sequence, together with a sequence of human NMT, which extends further upstream of that currently accepted [19,23,27] as the 5'-end of the human NMT mRNA (Figure 2). The sequence reported here is longer than this, but contains the sequence recently described for the human NMT1 gene [27]. Neither increasing either the stringency or the time of the first-strand cDNA synthesis, nor adjustments to the amplification conditions (to favour the amplification of longer-sized products), resulted in generation of cDNA longer than that seen in Figure 2.

As described in the Introduction, forms of NMT smaller in size than that produced from the extended sequence have been described and can be demonstrated in immunoblots of both cell lines and normal tissues. These could represent either fragments of the enzyme or the products of alternatively spliced mRNAs. Since the C-terminus of the enzyme is essential for activity [19,28], it seems probable that any such splice variants would be altered at the N-terminus of the enzyme. To determine if this is the case, amplification of the 5'-end of NMT mRNA was performed using specific primers, one (NMT-F_{out}) derived from the new 5'-sequence identified here and the other (NMT-R_{out}) based on the sequence within NMT. PCR using these primers resulted in the amplification of two cDNA products: the major product was of the predicted size (478 bp), but another approx. 110 bp shorter (Figure 1B) of lower abundance was also detected, though very faintly, in lymphocytes. The regions of the agarose gel corresponding in size to the short form of these amplified products were excised and reamplified with the nested primers, NMT-F_{in} and NMT-R_{in}. The resulting products had the predicted molecular sizes (Figure 1C), demonstrating the specificity of the amplification of the initial short product from the three different tissue sources.

Sequencing of the initial amplification products (367 bp)

confirmed their full identity with the NMT gene sequence, except for a 109 bp deletion (positions 157 to 265; Figure 2). This introduces a frameshift into the open reading frame of the NMT-L, which results in the generation of a stop codon upstream of the fourth methionine of the NMT-L (see the NMT-S sequence; Figure 2).

In order to compare NMT produced in human cells with that encoded by the plasmids containing the different forms of NMT, the latter were translated *in vitro* and compared with the NMT immunoprecipitated from [³⁵S]methionine-labelled HeLa and HEK 293 cells. The results showed that the NMT_L (63 kDa) produced from the newly extended sequence (NMT-L) was identical in size to the major specifically immunoprecipitated protein derived from both cell lines (Figure 3). This indicated that the full-length enzyme had been produced from the newly isolated sequence, whereas plasmids pNMT-M and pNMT-S produced proteins smaller than that produced by pNMT-L, but which had apparent molecular masses close to those predicted from their sequence. Both pNMT-M and pNMT-S produced minor amounts of smaller proteins, although this was less evident with pNMT-L (Figure 3). These could have been produced either by initiation of protein synthesis from internal in-frame methionines or by premature truncation of the protein sequence.

A specific protein could also be immunoprecipitated specifically from the cell lysates, which co-migrated with the major protein produced *in vivo* from pNMT-S. This might therefore represent the production of enzyme from the alternatively spliced NMT-S mRNA. The other specifically immunoprecipitated protein that co-migrates just below the 63 kDa major protein in the cells, and is especially detectable in HEK 293 cells, might be the product of the recently described second gene for NMT (NMT2). The protein bands that were electrophoresed with molecular masses significantly lower than 45 kDa were probably degradation products of NMT.

In order to determine if more than one form of NMT is present in different mammalian tissues, we have examined a range of rat tissues that can be processed rapidly for analysis by immunoblot. The results show that in most of the tissues, the affinity-purified antibodies to human NMT cross-react with a rat protein of molecular mass 62 ± 1.7 kDa ($\pm$ S.D., $n = 3$; Figure 4), which is similar in size to the HeLa enzyme. However, in brain and stomach, a smaller form of the enzyme could be found. This has an apparent molecular mass of 47.5 ± 2.1 kDa ($\pm$ S.D., $n = 3$), which is close to that predicted for NMT translated from the first in-frame ATG in the sequence of NMT-S described in this report, and could therefore represent translation of the enzyme from the alternatively spliced mRNA.

The results presented here indicate that the regulation of the synthesis of human NMT is more complex than was previously realised. During the course of this work, a second gene for human NMT (NMT2), differing only slightly in sequence from that described here, has been described [27]. Furthermore, in the same report, the start codon we show is the origin of NMT synthesis *in vivo* (initiating NMT_L; Figure 1) was also identified as the start site for translation of the enzyme from the NMT1 gene [27]. Transfection of COS cells with NMT1, but not with NMT2, resulted in a complex pattern of immunoreactive NMT bands, which the authors attributed to protein processing. The data presented here suggest that the appearance of the 48 kDa form of the enzyme in their experiments may reflect either the proteolytic cleavage of the overexpressed protein or the initiation of its translation from the internal fourth methionine. However, the production of the 48 kDa form of NMT *in vivo* may be derived from the alternative splicing of the NMT1 gene rather than from protein processing.

Figure 3 *In vitro* translation of the different clones of human NMT, and comparison of the translated proteins with those found in HEK 293 and HeLa cell lines

Samples (1 μ l) of the translation products from pNMT-L, pNMT-M and pNMT-S, together with immunoprecipitates from [35 S]methionine-labelled HeLa and HEK 293 cells, were analysed using SDS/PAGE (10% gels) and the position of the radioactive bands was determined by fluorography. The specific immunoprecipitates (NMT) were performed using 5 μ g of affinity purified anti-(human NMT), while the control immunoprecipitates (Con) were performed using the same amount of a pre-immune rabbit IgG. The latter contain a non-specific band, migrating more slowly than NMT, which is also found in the specific immunoprecipitates.

Figure 4 Immunoblotting of rapidly isolated rat tissues from liver, brain, spleen, heart, kidney, stomach and adrenal gland

Tissue extracts (50 μ g) were immunoblotted by SDS/PAGE (10% gels) using 100 ng/ml of affinity-purified rabbit anti-NMT antibody, followed by development of the blots using an alkaline-phosphatase-conjugated swine anti-rabbit antibody. The major NMT band at 62 kDa is clearly present in all tissues, although only faintly visible in the stomach sample. Both brain and stomach isolates also show a strongly immunoreactive band at 47.5 kDa, which could be derived from the alternatively spliced mRNA, and a faint band corresponding to this can also be seen in the kidney tissue sample. The immunoreactive band running at approximately 45 kDa in brain is a degradation product of NMT.

The tissue survey of normal rat tissues, prepared rapidly for analysis, does suggest that in some tissues, notably brain and stomach, there is a significant fraction of the total NMT present as a 48 kDa form, which could represent the alternatively spliced form of NMT. Since the 48 kDa form of NMT would lack the ribosomal targeting sequence [23], it would be expected to be predominantly cytosolic. This raises the possibility that the intracellular targeting of this enzyme is regulated by alternative mRNA splicing, and that this process may form part of its normal developmental or physiological control.

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