An Evolutionarily Conserved N-terminal Acetyltransferase Complex Associated with Neuronal Development*

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We previously identified mNAT1 (murine N-terminal acetyltransferase 1) as an embryonic gene that is expressed in the developing brain and subsequently down-regulated, in part, by the onset of N-methyl-D-aspartate (NMDA) receptor function. By searching the data base we discovered a second closely related gene, mNAT2. mNAT1 and mNAT2 are highly homologous to yeast NAT1, a gene known to regulate entry into the G0 phase of the cell cycle. However, in the absence of further characterization, including evidence that mammalian homologues of NAT1 encode functional acetyltransferases, the significance of this relationship has been unclear. Here we focus on mNAT1. Biochemical analysis demonstrated that mNAT1 and its evolutionarily conserved co-subunit, mARD1, assemble to form a functional acetyltransferase. The infection of mouse mNAT1 and mARD1 followed by immunofluorescent staining revealed that these proteins localize to the cytoplasm in both overlapping and separate compartments. In situ hybridization demonstrated that throughout brain development mNAT1 and mARD1 are highly expressed in areas of cell division and migration and are down-regulated as neurons differentiate. Finally, mNAT1 and mARD1 are expressed in proliferating mouse P19 embryonic carcinoma cells; treatment of these cells with retinoic acid initiates exit from the cell cycle, neuronal differentiation, and down-regulation of mNAT1 and mARD1 as the NMOA receptor 1 gene is induced. The results provide the first direct evidence that vertebrate homologues of NAT1 and ARD1 form an evolutionarily conserved N-terminal acetyltransferase complex and suggest that expression and down-regulation of this enzyme complex plays an important role in the generation and differentiation of neurons.

Over half of all eukaryotic proteins are N-terminally acetylated (1). A functional role for this highly prevalent post-translational modification has been demonstrated for the activity of two peptide hormones; the N-terminal acetyl group of α-melanocorticotropin is necessary for its melanocyte-stimulating activity (2), and N-terminal acetylation of β-endorphin abolishes its analgesic action (3). Apart from such rare exceptions, the biological significance of N-terminal acetylation in higher eukaryotes is unknown. In contrast, considerably more is understood about the role of N-terminal acetylation in Saccharomyces cerevisiae, where three N-terminal acetyltransferases that act on distinct groups of substrates have been identified (4). One of these enzymes, NatA, is a complex composed of two proteins, NAT1 and ARD1 (5). The nat1 null mutant displays slow growth, derepression of the silent mating locus HML, failure to enter G0, and failure to sporulate (6). An identical phenotype is displayed by strains with a null mutation of ard1 (7, 8). Therefore, it has been proposed that in yeast the NAT1/ARD1 complex is required to regulate some protein or proteins involved in cell cycle control (6).

Electrical activity mediated by the N-methyl-D-aspartate (NMDA) class of glutamate receptors in the developing brain has been shown to play a role in neuronal survival (9), migration (10), proliferation (11), the formation of precise neural circuits (12, 13), and has been implicated in fetal alcohol syndrome (14) and schizophrenia (15). Influx of calcium through activated NMDA receptors initiates intracellular signaling cascades that lead to changes in the activity of transcription factors such as cAMP-responsive element-binding protein and ultimately to changes in gene expression (16).

Moreover, in the adult hippocampus NMDA receptor-induced changes in gene expression are thought to be necessary for long term changes in synaptic efficacies (17). To identify molecular events regulated by NMDA receptor function in developing neurons, we used the disrupted whisker representation neural circuit in NMDAR1 knockout mice (18) as model for cDNA microarray analysis. Three genes were identified that are expressed at higher than normal levels in the absence of functional NMDA receptors (19). One of these, mNAT1, is highly homologous to the S. cerevisiae N-terminal acetyltransferase subunit NAT1 (6).

In addition to mNAT1, other vertebrate homologues of NAT1 have been identified in screen for molecules expressed very early in embryogenesis (20), as well as in developing blood vessels (21). Based on these observations, it has been suggested that these genes encode an N-terminal acetyltransferase that plays an important developmental role that is evolutionarily conserved from lower eukaryotes to mammals. However, in the absence of detailed developmental studies, full-length coding sequence for mammalian NAT1, and biochemical and functional evidence that it encodes an acetyltransferase, the viability of this hypothesis has been unclear. Here we demonstrate for the first time that mammalian NAT1 and mammalian

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF510858.

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‡ The abbreviations used are: NMDA, N-methyl-D-aspartate; NMDAR, NMDA receptor; E, embryonic day; P, postnatal day; m, mouse; HA, hemagglutinin; MT, Myc-tagged; EST, expressed sequence tag; PBS, phosphate-buffered saline; RIPA, radiimmunoprecipitation assay.
ARD1 combine to form a functional acetyltransferase. In addition, we show that mNAT1 and mARD1 are expressed in regions of cell division and migration throughout brain development and are down-regulated as neurons mature and form appropriate patterns of synaptic connections. This inverse correlation between mARD1/mNAT1 expression and neuronal maturation is recapitulated in retinoic acid-stimulated P19 embryonic carcinoma cells that form undifferentiated neuronal differentiation in vitro. The results suggest that a N-terminal acetyltransferase complex containing mNAT1 and mARD1 forms an evolutionary link between developmental mechanisms in lower eukaryotes and those used in the mammalian brain.

EXPERIMENTAL PROCEDURES
cDNA Clones and Expression Constructs—The complete cDNA sequence for mNAT1 was deduced from two overlapping cDNA clones, 3a and 8f, as well as from three ESTs: AA561496, AA163290, and AA474587 (see Fig. 1A). To obtain 3A and 8F, PCR primers were designed based on EST AA474587, and these were used to screen an arrayed mouse testis cDNA library (Origene). It should be noted, however, that 3A and 8F are conventional cDNA clones. The coding sequence in clone 3A was sequenced on both strands. The complete mNAT1 cDNA sequence has been registered in the GenBank™ data base (accession number AF510858).

An intermediate construct, pCMV6-mNAT1, that contains full-length mNAT1 cDNA, was assembled by a three fragment ligation of an 1.99-kb PstI/BamHI fragment from clone 3A, and the BamHI/EcoRI (blunted) fragment from clone 8F. Construct CS2+MT-mNAT1, used for cellular expression and localization of Myc-mNAT1 (see Fig. 5 and Fig. 6C), was generated by subcloning the 1.53-kb SpeI/PspI (blunted) fragment from CMV6-mNAT1 between the XbaI and SnaBI sites of the CS2+MT vector (22, 23). Subsequently, the XhoI (blunted)/SpeI fragment of this construct was replaced with the 1.26-kb SpeI/PspI (blunted) fragment from CMV6-mNAT1 to obtain CS2+MT-mNAT1. A construct used for in vitro translation of mNAT1, as well as some acetyltransferase assays (see Fig. 6A), pT7-mNAT1, was generated by replacing the luciferase cDNA in the luciferase T7 control DNA (Promega) with the NotI/DraI fragment from pCMV6-mNAT1. A second construct used for in vitro translation of mNAT1 and the remainder of the acetyltransferase assays (see Fig. 6B), pT7-MT-mNAT1, was assembled by subcloning the NotI (blunted)/SpeI fragment from CS2+MT-mNAT1 into the NotI (blunted)/SpeI sites of pT7-mNAT1.

A cDNA clone representing mARD1 was identified in the GenBank™ data base (accession number NM_019870) by searching for sequences homologous to yeast ard1. The 5′-SSS-base pair mARD1 cDNA was obtained by reverse transcription PCR (RT-PCR) using postnatal day 0 (P0) mouse embryonic carcinoma cells that are undergoing neuronal differentiation in retinoic acid-stimulated P19 embryonic carcinoma cells (Asahi Techno Glass Corp., Funabashi, Japan). These aggregate cultures were maintained 4 days (12 h, 1 day, and 4-day time points in Fig. 4), transfected, and plated on tissue culture dishes without retinoic acid. Two days later, the medium was replaced with fresh medium containing 5 μg/ml cytosine arabinoside (Sigma); the culture was maintained for up to another 6 days for a total of 8 days in culture after retinoic acid treatment (4 + 4-day and 4 + 8-day time points in Fig. 4). Total RNA was extracted from the cells using Trizol (Invitrogen).

Nase Protection—RNA extraction and RNase protection analysis was carried out as described previously (19, 27). The dried gels were exposed at −70 °C to Eastman Kodak Co. BioMax MS film, and signals were quantified directly from the gels using an Amersham Biosciences Storm 860 PhosphorImager. Riboprobes were used as follows: mNAT1, nucleotides 577–867 of NM_019870; mARD1, nucleotides 357–787 of NM_019870 plus an additional 91-nucleotide insertion (extra exon) 5′ to nucleotide 622; NMDAR1, nucleotides 994–1314 of U08261; NMDAR1 knockout pups (18) were generated by heterozygous crosses, and genotyping was performed as described (19).

Cellular Localization of mARD1 and mNAT1—Rat NRK-49F cells were maintained in Dulbecco’s modified Eagle’s medium containing 5% fetal bovine serum, and 6 × 10⁴ cells were replated on polyl-t-lysine-coated coverslips the day before transfection. Equimolar amounts (0.5 μg each) of CS2+MT-mNAT1 and CS2+MT/mARD1/HA were transfected into the cells using FuGENE 6 transfection reagent (Roche Applied Science). After 36 h, the cells were fixed and permeabilized by incubating them in cold ethanol for 10 min and blocked with 0.1% cold serum in PBS for 60 min. For HA immunostaining, 3% F10 rat anti-HA monoclonal antibodies (Roche Applied Science) were used at 0.7 μg/ml and followed by an Alexa488-conjugated goat anti-rat polyclonal antibody (Molecular Probes) at a dilution of 1:500. For Myc immunostaining, 9E10 mouse anti-c-Myc monoclonal ascites fluid (Sigma) was used at a dilution of 1:1,000, followed by Cy3-conjugated donkey anti-mouse antibody (Jackson ImmunoResearch) at a dilution of 1:1,000. Samples were examined using a Zeiss LSM410 laser-scanning confocal microscope with a ×63 oil immersion objective. Results presented in Fig. 5 are representative of four independent experiments; for one of these, the four cells were fixed with 4% paraformaldehyde in PBS for 15 min, permeabilized with 0.1% Triton X-100 in PBS for 30 min, and blocked in 10% calf serum in PBS for 10 min, rather than the cold methanol treatment described above. Similar results were obtained with both methods.

Immunoprecipitation—In vitro translation was performed in the presence of Redivue [35S]methionine (Amersham Biosciences) using a TNT T7 Quick-coupled transcription/translation system (Promega). mNAT1 and HA-tagged mARD1 proteins were assembled using constructs pT7-mNAT1 and pBS-mARD1/HA, respectively.

Co-immunoprecipitation of in vitro translated protein was carried out by incubating 25 μl of the mNAT1 translation reaction, 10 μl of the mARD1/HA translation reaction, 20 μl of 250 μg/ml rabbit anti-HA antibody (Zymed Laboratories Inc.), and 45 μl of RIPA buffer (10 mM Tris, pH 7.4, 2 mM EDTA, 0.15 M NaCl) on ice for 100 min. At this point 130 μl of RIPA buffer and 20 μl of protein A-Sepharose (Zymed Laboratories Inc.) were added, and the reaction was incubated on ice for 2 h. The protein A-Sepharose beads were then washed four times with RIPA buffer containing 1% Nonidet P-40 and resuspended in 30 μl of 1 × SDS-PAGE sample buffer, and 20 μl were resolved on an 11% SDS-PAGE gel.

Co-immunoprecipitation from transfected mammalian cells was carried out as follows. Human embryonic kidney 293 cells were plated on 35-mm dishes 24 h before transfection. Cells at 50% confluency were transiently transfected with 0.75 μg each of CS2+MT-mNAT1 and CS2+ARD1/HA plasmids using FuGENE 6 transfection reagent (Roche Applied Science). After 2 days, the 293 cells were harvested in 1 ml of RIPA buffer containing 1% Nonidet P-40 and 1 mM phenylmethylsulfonyl fluoride. The solution was passed through a 21.5-gauge needle three times and incubated on ice for 30 min. This lysate was centrifuged, and the supernatant was precleared by incubating with rabbit IgG and blocked with 10% calf serum in PBS at room temperature. The precleared lysate was incubated with 1 μl of rabbit anti-HA (Zymed) and 1 μl of rabbit anti-Myc (Zymed), and the immunoprecipitate was detected using a standard Western blotting procedure. Briefly, 12 μl of the sample was resolved on a 7.5% SDS-PAGE gel, transferred to an Immobilon-P membrane (Millipore), and probed with anti-Myc monoclonal antibody 9E10 (Zymed Laboratories Inc.). The blot was visualized using horseradish peroxidase-conjugated...
Acetyltransferase Assay—Acetyltransferase activity determinations (see Fig. 6, A and B) were performed in triplicate. Briefly, in vitro translation of mNAT1 and HA-tagged mARD1 was carried out as described above. For immunoprecipitation, 60 µl of mNAT1 translation mixture, 20 µl of mARD1 translation mixture, 15 µg of rabbit anti-HA antibody (Zymed Laboratories Inc.), 160 µl of RIPA buffer, and 30 µl of anti-mouse IgG antibody (Chemicon) and the Chemilucent Western blot detection system (Chemicon).

Acetyltransferase Assay—Acetyltransferase activity determinations (see Fig. 6, A and B) were performed in triplicate. Briefly, in vitro translation of mNAT1 and HA-tagged mARD1 was carried out as described above. For immunoprecipitation, 60 µl of mNAT1 translation mixture, 20 µl of mARD1 translation mixture, 15 µg of rabbit anti-HA antibody (Zymed Laboratories Inc.), 160 µl of RIPA buffer, and 30 µl of anti-mouse IgG antibody (Chemicon) and the Chemilucent Western blot detection system (Chemicon).
protein A-Sepharose were incubated on ice for 16 h. The complex bound to the Sepharose was separated by centrifugation and washed three times with RIPA buffer containing 1% Nonidet P-40 and once with RIPA buffer without Nonidet P-40. N-terminal acetyltransferase activity was then assayed as described previously (28, 29), with some modifications. Briefly, the Sepharose-bound complex was incubated at 30 °C for 3 h in a 150-μl reaction containing 0.2 mM potassium phosphate, pH 8.0, 4.5 mM [3H]acetyl-CoA (137 GBq/mmol; Amersham Biosciences), and 33 μM human adrenocorticotropic hormone (ACTH 1–24; Calbiochem) with constant agitation. The reaction was then centrifuged, and 130 μl of supernatant was applied to 150 μl of SP Sephadex (50% slurry in 0.5 M acetic acid; Sigma). The resin was washed three times with 1 ml of 0.5 M acetic acid and rinsed with 300 μl of methanol, and radioactivity was determined by scintillation counting.

RESULTS

Vertebrate Homologues of Yeast NAT1—We previously identified EST AA474587, a partial cDNA clone corresponding to the 3′-untranslated region of mNAT1, because it is expressed at higher than normal levels in the developing brains of NMDAR1 knockout mice (19). To obtain full-length cDNA corresponding to mNAT1, we screened a mouse testis cDNA library and isolated clone 3A. Sequence analysis indicated that this clone lacked the 5′ end of the coding region, which was obtained from EST AA163290 and EST AA561496 (Fig. 1A). An open reading frame of 2,595 nucleotides encoding an 865-amino acid protein was deduced from the assembled sequence (Fig. 1B). mNAT1 and yeast NAT1 share 28% identity and 41% similarity when conservative amino acid substitutions are included.

One additional clone, 8F, was found to be identical in sequence to that assembled from 3A, AA163290, and AA561496, with the exception of the 5′-most region and a silent polymorphism observed at nucleotide 1874 of the registered cDNA sequence AF510858 (G → A). Comparison of the 5′ region to the corresponding mouse genomic sequence indicates that 8F represents an mRNA that resulted from alternative usage of an upstream exon (data not shown). This alternative splicing would yield a protein that is 50 amino acids shorter at the N terminus than the one predicted by the other cDNAs we analyzed.

A search of the GenBank™ data base identified a cDNA (accession number AK005056) that encodes a mouse protein that is 68% identical to mNAT1 (Fig. 1B). When conservative amino acid substitutions are included, the overall similarity is 81%. Northern blot analysis of mNAT2 indicated that, in the adult, highest levels of expression are present in the kidney and testes (Fig. 1C). Moderate expression was observed in the liver, thymus, and skin. Although the expression profiles of mNAT1 and mNAT2 are generally similar, expression levels of mNAT2 are lower than those of mNAT1, in particular in the brain (Fig. 1C; see also Ref. 19). Therefore, the present analysis focused on mNAT1.

A Vertebrate Homologue of Yeast ARD1—Studies in S. cerevisiae demonstrated that, to form a functional N-terminal acetyltransferase complex, NAT1 must assemble with another gene product, ARD1 (5). Although the mouse homologue of ARD1 has not been reported, we found a mouse cDNA in GenBank™ (accession number NM_019870) that encodes a protein that shares 42% identity with yeast ARD1. Based on this sequence, primers were designed to amplify mouse ARD1 cDNA. Reverse transcriptase PCR resulted in two products of ~291 and 382 base pairs, respectively. Sequence analysis of these cDNAs revealed that the shorter of the two is identical to NM_019870, whereas the longer one appears to be derived from an alternatively spliced mRNA with an extra 91 nucleotides inserted 5′ to nucleotide 622 of NM_019870. Comparison of these two cDNA sequences with the corresponding mouse genomic sequence suggests that they resulted from an alterna-
mNAT1 is expressed at high levels throughout the neural tube of the E11.5 brain, which is dominated by proliferating and migrating cells. These active cell populations also make up the bulk of the brain at E13 and E17, when mNAT1 expression remains high. By P0 mNAT1 expression has decreased in caudal regions of the brain, e.g. superior colliculus, tegmentum, and medulla, where neurons are relatively differentiated, and mitotic and migratory activities have subsided in comparison to earlier ages. In contrast, expression is maintained at relatively high levels in the olfactory bulb, neocortex, ventricular zone, and hippocampus, regions where cell division and migration are still prominent in the neonate. By P7 mNAT1 expression is low throughout most of the brain, although significant expression is maintained in the hippocampus and the cerebellar cortex. Overall expression at P40 is similar in pattern to that observed at P7 but slightly higher.

The expression pattern of mARD1 is similar to that observed for mNAT1. Like mNAT1, mARD1 is expressed throughout the developing brain from E11.5 through E17. However, it is expressed at lower levels and in a somewhat diffuse pattern, especially at E13 and E17. Unlike mNAT1, mARD1 continues to be expressed throughout the brain at P0. Expression then decreases and appears in a pattern resembling that observed for mNAT1 at P7 and P40, with overall low levels of expression and somewhat higher levels observed in the olfactory bulb, hippocampus, and superficial layers of the cerebellar cortex.

Down-regulation of mNAT1 and mARD1 in P19 Cells Undergoing Neuronal Differentiation—Mouse P19 embryonic carcinoma cells are multipotential cells that can be induced to exit the cell cycle and attain a neuron-like phenotype by culturing them as an aggregate in the presence of retinoic acid (26). During neuronal differentiation, P19 cells start to express various neuronal markers including glutamic acid decarboxylase (30), NCAM (31), NMDA receptors, and metabotropic glutamate receptors (32, 33). We extracted RNA from P19 cells at various stages of differentiation and used RNase protection to measure changes in mRNA levels for NMDAR1, mNAT1, and mARD1. As in previous reports (32, 33), NMDAR1 was not detected in untreated P19 cells and is induced as differentiation proceeds (Fig. 4). In contrast, mNAT1 and mARD1 are expressed at highest levels before the addition of retinoic acid. Despite the large number of cells that don’t differentiate upon

**Fig. 3. Spatiotemporal regulation of mNAT1 and mARD1 during mouse brain development.** In situ hybridization results are presented in darkfield optics for the indicated ages. Controls are presented for mNAT1 on E11.5, E13, and E17 samples; note that sense controls were performed for both probes at all ages analyzed, and comparable low levels of background were observed in each case. Cx, cortical neuroepithelium; L, lateral ventricle; Sc, superior colliculus; 4, fourth ventricle; To, tongue; M, medulla; S, spinal cord; Cp, cortical plate; Ob, olfactory bulb; Aq, cerebral aqueduct; Vz, ventricular zone; H, hippocampus; T, thalamus. Scale bar, 1 mm.
Neuronal differentiation of P19 cells was induced with 1 μM retinoic acid (RA) as described under “Experimental Procedures.” The cells were harvested at the indicated times for RNA extraction. For RNase protection, riboprobe complementary to mNAT1, mARD1, and NMDAR1 mRNA were synthesized, isolated, annealed with 5 μg of the indicated total RNA sample, digested with RNases, and fractionated by gel electrophoresis. Autoradiographs of gels are shown, with the sizes of undigested probes (arrowheads), as well as protected species (arrows), indicated. The t = 0 sample was not treated with retinoic acid (~RA). Probe, undigested probe; tRNA, negative control.

Cellular Localization of mNAT1 and mARD1—Rat kidney fibroblast cells (cell line NRK-49F) were transiently transfected with Myc-tagged mNAT1 and HA-tagged mARD1. After fixation, the cells were incubated with mouse anti-Myc and rat anti-HA monoclonal antibodies, followed by appropriate secondary antibodies labeled with either Alexa488 (anti-rat) or Cy3 (anti-mouse). Cellular localization of mNAT1 and mARD1 was then determined by immunofluorescence confocal microscopy (Fig. 5). Both proteins appear in the cytoplasm and are excluded from the nucleus. Although some overlap in distribution was observed, they are present in largely separate compartments. mNAT1 is found close to the nucleus, in a perinuclear pattern. mARD1 is present from the peripheral cytoplasm to regions close to the nucleus, where in some cells it formed a concentric ring (e.g. Fig. 5B). Overlap of mNAT1 and mARD1 distribution occurred in this most proximal region of mARD1 signal (Fig. 5, F and I).

An mNAT1/mARD1 Complex With N-Terminal Acetyltransferase Activity—mNAT1 and HA-tagged mARD1 were translated separately in vitro, and the resulting products were mixed together. To separate any mNAT1/mARD1 complex from the reticulocyte lysate, which may contain endogenous acetyltransferase activity, epitope-tagged mARD1 was immunoprecipitated using an anti-HA antibody. SDS-PAGE analysis of the resulting precipitate confirmed that mNAT1 assembles with mARD1 to form a complex (Fig. 6A). Acetyltransferase activity was measured using [3H]acetyl-CoA and adrenocorticotropic hormone, a peptide of 24 amino acids that has been used in previous N-terminal acetyltransferase assays (28). The mNAT1/mARD1 complex displayed acetyltransferase activity more than 10-fold above background (Fig. 6A). In contrast, little activity was present in controls samples in which ARD1 was omitted or the anti-RA antibody was replaced with IgG.

To determine whether mNAT1 requires mARD1 to form a functional acetyltransferase, as is the case for yeast NAT1 (5), we replaced mNAT1 with a Myc-tagged mNAT1 (MT-NAT1) that could be assayed for acetyltransferase activity independent of mARD1 (Fig. 6B). Although MT-NAT1 and mARD1 formed an active acetyltransferase, MT-NAT1 alone did not (Fig. 6B). The results strongly suggest that mNAT1 requires the presence of mARD1 to be functional.

Finally, a critical issue regarding the physiological relevance of mNAT1 and mARD1 is whether these proteins co-assemble in actual cells. Therefore, human embryonic kidney 293 cells were transfected with HA-tagged mARD and Myc-tagged mNAT1. HA-tagged mARD1 was immunoprecipitated from the cell lysate 24 h later using an anti-HA antibody. Western blotting was performed using an anti-Myc antibody to determine whether mNAT1 co-assembled with mARD1. The presence of Myc-tagged mNAT1 on the Western blot (Fig. 6C, lane 2) demonstrates that mNAT1 and mARD1 co-assemble in mammalian cells.

**DISCUSSION**

Brain development is characterized by large changes in the size, shape, and location of individual neural precursors as they exit the cell cycle, migrate from the ventricular zone, and become highly specialized mature cells. Molecular events sur-
Fig. 6. mNAT1 and mARD1 co-assemble to form a functional acetyltransferase. A, mNAT1 (NAT1) and HA-tagged mARD1 (ARD1-HA) were translated separately in vitro in the presence of \(^{35}S\)methionine to form 90- and 45-kDa proteins, respectively (Input). The amount of protein seen in each input lane represents 20% of the amount of protein used in each immunoprecipitation reaction. Anti-HA antibody immunoprecipitated (IP) both mNAT1 and mARD1 when the two were mixed, indicating that they co-assemble in vitro. No immunoprecipitation of either protein occurred when IgG was substituted for the anti-HA antibody or when HA-tagged AD1 was omitted from the reaction. Acetyltransferase activity was determined by incubating each immunoprecipitate with \(^{3}H\)acetyl-CoA and adrenocorticotrophic hormone peptide. The acetylated peptide was isolated by cation-exchange chromatography and counted for radioactivity. B, mNAT1 is not functional in the absence of mARD1. Myc-tagged mNAT1 (MT-NAT1) and ARD1-HA were translated separately in vitro in the presence of \(^{35}S\)methionine (Input). Anti-MT (shown) and anti-HA antibodies immunoprecipitated both mNAT1 and mARD1 (IP). No immunoprecipitation of either protein occurred when IgG was substituted for the anti-MT or anti-HA antibodies. When mARD1 is present, MT-NAT1 has acetyltransferase activity; MT-NAT1 alone displays only background acetyltransferase activity, as well as the substrate specificity of mNAT1/mARD1 and the putative mNAT2/mARD1, compare? In addition, in yeast, NAT1 must assemble with ARD1 to be functional (5). To begin to answer the question of whether the contribution of both mNAT1 and mNAT2 be taken into consideration.

Co-expression of mNAT1 and mARD1 in a rat kidney fibroblast cell line revealed overlapping but largely separate localization of these two proteins. Both are present in the cytoplasm and excluded from the nucleus, but mNAT1 is distributed in a perinuclear pattern, whereas mARD1 is present from the peripheral cytoplasm to regions close to, but generally not adjacent to, the nucleus. The extent of non-overlap of the distribution of these two proteins raises questions regarding function and regulation. For example, is mNAT2 present in the cell in regions where mNAT1 is absent? How might the levels of acetyltransferase activity, as well as the substrate specificity of mNATURE/mARD1 and the putative mNAT2/mARD1, compare? In addition, in yeast, NAT1 must assemble with ARD1 to be functional (5). To begin to answer the question of whether the region of overlap is the only region in the cell where mNAT1, combined with mARD1, forms functional N-terminal acetyltransferase, we used in vitro translation combined with an acetyltransferase assay. In contrast to what has been reported for a biochemically purified rat N-terminal acetyltransferase that shares a number of features with yeast NAT1 (29), including approximate size as well as substrate specificity, we found no acetyltransferase activity associated with mNAT1 in the absence of mARD1. It remains possible that the Myc tag on the N terminus of mNAT1 eliminates its autonomous acetyltransferase activity but not its activity when combined with mARD1. However, a more likely explanation is that mNAT1, like yeast NAT1, is not functional on its own.

mNAT1 and mARD1 are expressed at relatively high levels in neural precursors and down-regulated during neuronal development. This is the case in the brain, as well as in mouse embryonic carcinoma P19 cells treated with retinoic acid, which causes these cells to differentiate and express neuronal markers, including NDMA receptors. It is interesting that a
certain amount of mNAT1 and mARD1 expression remains in P19 cultures at the conclusion of the experiments, i.e. 12 days after the initiation of retinoic acid treatment. One possible explanation is that expression of these genes is maintained in a population of undifferentiated cells that do not respond to retinoic acid (26). Alternatively, some expression of mNAT1 and mARD1 may be maintained, because differentiation of retinoic acid-treated P19 cells is incomplete, and they are neither progenitors nor fully differentiated neurons. In the brain, an exception to the overall pattern that mNAT1 and mARD1 are down-regulated during development is found in the hippocampus, where adult levels of expression of both genes are as high as those observed at earlier times. Interestingly, the hippocampus is also a site of neurogenesis and synaptic plasticity in the adult. Moreover, some expression of mNAT1 is evident in the adult cerebellar cortex, another region of synaptic plasticity. Taken together, the results are consistent with the possibility that mNAT1 is a marker for cells that are immature, retain the ability to divide, or can undergo long term changes in synaptic structure or function.

The present study provides the first direct evidence that mammalian NAT1 and mammalian ARD1 combine to form a functional and evolutionarily conserved N-terminal acetyltransferase. However, it is not yet clear to what extent the evolutionary relationship between these proteins and their yeast predecessors translates into conserved developmental significance for the cell. For example, yeast NAT1 regulates yeast predecessors translates into conserved developmental evolutionary relationship between these proteins and their transferase. However, it is not yet clear to what extent the significance of mNAT2.

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