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Molecular Identification of Aspartate N-acetyltransferase and its Mutation in Hypoacetylaspartia

Elsa WIAME*, Donatienne TYTECA[†], Nathalie PIERROT[§], François COLLARD*, Mustapha AMYERE[‡], Gaëtane NOEL*, Jonathan DESMEDT^{||}, Marie-Cécile NASSOGNE^{||}, Miikka VIKKULA[‡], Jean-Noël OCTAVE[§], Marie-Françoise VINCENT^{||}, Pierre J. COURTOY[†], Eugen BOLTSHAUSER[¶] and Emile VAN SCHAFTINGEN*¹

*Université Catholique de Louvain and de Duve Institute, Laboratory of Physiological Chemistry and [†]CELL Unit and [‡]Laboratory of Human Molecular Genetics, B-1200 Brussels, Belgium

[§]Université Catholique de Louvain, Institute of Neuroscience, Experimental Pharmacology Unit, B-1200 Brussels, Belgium

^{||}Cliniques Universitaires Saint-Luc, Laboratory of Metabolic Diseases and [¶]Department of Pediatrics, Pediatric Neurology Unit, B-1200 Brussels, Belgium

[¶]University Children's Hospital of Zurich, Department of Pediatric Neurology, Zurich, Switzerland

¹Corresponding author: Emile Van Schaftingen (Tel.: 003227647564; Fax: 003227647598; E-mail: emile.vanschaftingen@uclouvain.be).

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SYNOPSIS

The brain-specific compound N-acetylaspartate (NAA) occurs almost exclusively in neurons, where its concentration reaches ≈ 20 mM. Its abundance is determined in patients by magnetic resonance spectroscopy to assess neuronal density and health. The molecular identity of the N-acetyltransferase that catalyses NAA synthesis has remained unknown, because this enzyme is membrane-bound and difficult to purify. Database searches indicated that, among the putative N-acetyltransferases (i.e., proteins homologous to known N-acetyltransferases, but with uncharacterized catalytic activity) encoded by the human and mouse genomes, two, NAT8L and NAT14, were almost exclusively expressed in brain. Transfection studies in HEK293T cells indicated that NAT8L, but not NAT14, catalysed the synthesis of NAA from L-aspartate and acetyl-CoA. The specificity of NAT8L, its K_M for aspartate and its sensitivity to detergents are similar to those described for brain aspartate N-acetyltransferase. Confocal microscopy analysis of CHO cells and neurons expressing recombinant NAT8L indicates that it is associated with the endoplasmic reticulum, but not with mitochondria. Mutation search in the NAT8L gene in the only patient known to be deficient in NAA disclosed the presence of a homozygous 19-bp deletion resulting in a change in reading frame and the absence of production of a functional protein. We conclude that NAT8L, a neuron-specific protein, is responsible for NAA synthesis and is mutated in primary NAA deficiency (hypoacetylaspartia). The molecular identification of this enzyme opens perspectives for the clarification of the function of this most abundant amino acid derivative in neurons and for the diagnosis of hypoacetylaspartia in other patients.

Keywords : N-acetylaspartate, N-acetyltransferase, hypoacetylaspartia, membrane proteins, endoplasmic reticulum

INTRODUCTION

N-acetylaspartate (NAA), a brain-specific molecule discovered more than 50 years ago, is the second most abundant metabolite in brain after glutamate. It is essentially present in neurons, where its concentration reaches ≈ 20 mM. Its abundance can be determined by MRS (magnetic resonance spectroscopy) to monitor neuronal density and health in neurological disorders, including Alzheimer's disease, multiple sclerosis and AIDS encephalopathy [1]. NAA is formed from aspartate and acetyl-CoA by aspartate N-acetyltransferase (Asp-NAT), a membrane-bound enzyme [2-4], which is unstable in the presence of detergents [5] and could therefore not be purified. Its subcellular localization has been variously reported as mitochondrial [6,7] or microsomal [4,8]. NAA is not degraded in neurons, but it is transferred by an undefined mechanism to oligodendrocytes, the myelin-forming cells, where it is hydrolyzed by aspartoacylase (aminoacylase II). The released acetate serves as a precursor for the synthesis of fatty acids, required for the formation of myelin lipids [9]. NAA serves also as a precursor for N-acetylaspartylglutamate [10,11], the most abundant dipeptide present in brain and possibly a neurotransmitter [12]. Other proposed roles for NAA are osmoregulation or disposal of amino groups resulting from glutamate transamination [1]. Except that NAA does not appear to be a neurotransmitter [13], no agreement exists on the relative importance of these roles.

NAA metabolism is disrupted in two inborn errors of metabolism. Deficiency of aspartoacylase, caused by mutations in the *ASPA* gene, gives rise to a leukodystrophy known as Canavan disease (MIM 271900), which is characterized by onset in early infancy, dystonia, severe mental defect, blindness, megalencephaly, and death by 18 months on the average. [14]. NAA concentration is raised in brain, CSF, and urine. The 'opposite' disorder, i.e. the absence of brain NAA, has been described in only one patient, with truncal ataxia, marked developmental delay, seizures and secondary microcephaly [15-17]. This disorder, designated 'hypoacetylaspartia', is presumably due to a deficiency of Asp-NAT, but this has not been demonstrated.

A better definition of the role of NAA and the identification of the defect underlying hypoacetylaspartia would greatly benefit from the molecular identification of Asp-NAT. This identification was the purpose of the present work.

EXPERIMENTAL

Preparation of expression vectors

Human and mouse total brain cDNA were synthesized with random primers and 1.5-2.5 µg total RNA with the M-MuLV reverse transcriptase from Fermentas (St Leon-Rot, Germany) according to the manufacturer's instructions. FirstChoice[®] human brain total RNA was from Applied Biosystems and mouse total brain RNA was prepared as described in [18].

The open reading frames of mouse *NAT14* (UCSC refseq gene NM_201355) and mouse *NAT8L* (UCSC refseq gene NM_001001985) were PCR-amplified using 2.5 U of PWO polymerase (Roche Applied Science) in the presence of 1.5 M betaine (Sigma-Aldrich) with mouse total brain cDNA as a template. For the non-tagged constructions, a 5'-primer (for primer sequences, see Table S1 in the Supplementary data) containing the putative ATG preceded by a perfect Kozak sequence and a *KpnI* site, and a 3'-primer containing the putative stop codon flanked by a *XbaI* site were used. For the C-tagged constructs, the 5'-primers were the same, while the putative stop codon in the 3'-primers was mutated and flanked by a *XbaI* site. For the N-tagged constructs, the 5'-primers were devoid of Kozak sequence and the 3'-primers were the same as for the wild-type amplifications. The PCR amplifications started with an initial denaturation step at 96°C for 3 min, followed by 35 cycles for 30 sec at 96°C, 1 min at 60°C and 1 min at 72°C, and ended with a final extension step at 72°C for 5 min. All PCRs reported in this paper were performed with this 5-step program except for site-directed mutagenesis (see below). PCR products of the expected size were obtained, subcloned into pBlueScript plasmid and checked by sequencing. The *KpnI/XbaI* digested products were prepared from *E. coli* XL1blue strain (C-tagged constructs) or from *E. coli* JM110 strain (wild-type and N-tagged constructs) and inserted in pCMV5, pEF6MycHisA and pEF6HisB expression vectors for the wild-type, C-tagged and N-tagged constructs, respectively. For human NAT8L, amplifications were performed with 2.5 U of Pfu polymerase (Fermentas) in the presence of 1 M betaine, with 2 µl of human total brain cDNA as a template. The three types of construct were prepared as for the mouse sequences and checked by sequencing.

Site-directed mutagenesis

Fifty ng of the pBlueScript vector containing the human NAT8L sequence designed to be expressed with a polyHis tag at its C-terminus were used as a template, in a 50 µl-PCR reaction containing 25 pmol of each primer (Table S1), 0.2 mM dNTP, 1 M betaine, and 2.5 U Pfu polymerase in 1 X Pfu buffer. All these primers were phosphorylated at their 5' end. This mixture was subjected to thermal cycling at 96°C for 30 sec for denaturation, followed by 30 sec at 55°C for annealing, and 5 min at 72°C for extension. A total of 20 cycles was used and the procedure was ended by 7 min at 72°C. For both mutants, 3 identical reactions were done and pooled at the purification step in 35 µl of 10 mM TrisHCl, pH 8.5. The purified PCR product (30 µl) was incubated overnight at 16°C with 5 U of T4 DNA ligase (Fermentas) in the presence of 5 % PEG 4000 in 50 µl of 1 X ligase buffer. The ligation product was then digested for 2 hours at 37°C with 20 U of *DpnI* in order to eliminate the wild-type template. Fifteen µl of this mixture were used to transform 100 µl of *E. coli* XL1blue bacteria. The complete NAT8L coding region of all plasmids was sequenced to verify the introduced deletion and the absence of random mutations. The *KpnI/XbaI* fragment was then subcloned in pEF6MycHisA vector and both mutants were expressed as polyHis/myc-tagged fusion proteins.

Transfections of HEK 293T cells and western blotting

HEK 293T cells were cultured and transfected essentially as described by Rzem *et al.* [19] using the jetPEI procedure. After 48 h at 37°C, the cells were washed once with 5 ml of cold phosphate-buffered saline (PBS). They were scraped in 0.8 ml of 20 mM Hepes (pH 7.1) containing 5 µg/ml leupeptin and antipain, frozen in liquid nitrogen, thawed and lysed by vortexing. The extracts were incubated with 100 U/ml DNase I (from Sigma-Aldrich; prepared in 5 mM MgSO₄) for 1 hour on ice before use. Protein concentration was determined with the Bradford assay [20], using gamma-globulin as a standard. Western blots were performed as described [19] with a monoclonal pentaHis antibody (Qiagen) diluted 1:2000 in PBS containing 1 % bovine serum albumin. After washing, membranes were probed for 1 hour at room temperature with peroxidase-conjugated anti-mouse antibody. Detection was performed using the ECL Western blotting analysis system from GE Healthcare according to the manufacturer's protocol.

N-acetylaspartate measurements by GC/MS

Quantification of NAA in cell media was performed by isotope dilution gas chromatography-mass spectrometry as described by Kelley and Stamas [21] with *N*-[d₃]-acetylaspartate from VU Medical Center, Metabolic Laboratory (Dr H.J. ten Brink, Amsterdam, The Netherlands). Samples (0.2 ml) were treated with hydroxylamine hydrochloride in the presence of *N*-[d₃]-acetylaspartate as internal standard, and extracted with ethyl acetate followed by diethyl ether. After evaporation of the combined extracts under a stream of nitrogen, the residue was resuspended in acetonitrile, and derivatization was achieved with *N,O*-bis(trimethylsilyl)trifluoroacetamide/trimethylchlorosilane (BSTFA/TMCS). Analysis was performed by gas-chromatography (Hewlett-Packard 6890 series GC system equipped with a 30 m x 0.25 mm fused silica capillary column CP-SIL 8CB, Varian) with helium as carrier gas, coupled with a Hewlett-Packard 5973 Mass selective detector under electron-impact fragmentation. Mass spectrometric data were collected in the selected ion mode at *m/z* = 274 for native NAA, and *m/z* = 277 for synthetic isotopic NAA. Calibration curves were constructed by mixing 4.5 µg of the isotopic NAA with standard solution containing 0 – 6 µg of NAA, followed by drying under nitrogen and direct derivatization with the silylating agent.

Assay of aspartate N-acetyltransferase

Asp-NAT was assayed radiochemically in a mixture (200 µl final volume) comprising, unless otherwise stated 10 mM potassium phosphate, 20 mM Hepes, pH 7.1, 1 mM MgCl₂, 50 µM L-aspartate, 10⁵ cpm [U-¹⁴C]L-aspartate (GE Healthcare), 200 µM acetyl-CoA (Sigma-Aldrich), and 10 µl of a cell extract containing ≈ 5-10 mg protein/ml. After 20-40 min at 30°C, the reaction was arrested by a 5-min incubation at 80°C and 1 ml of 5 mM Hepes, pH 7.1 was added. The sample was centrifuged and the supernatant was applied on a 1-ml Dowex AG1X8 column (Cl⁻ form, 100-200 mesh, from Acros Organics) prepared in a Pasteur capillary pipette. The latter was washed successively with 2 ml 5 mM Hepes, pH 7.1, 5 ml 150 mM NaCl in the same buffer to elute unreacted aspartate, and 5 ml 300 mM NaCl to elute NAA. Radioactivity was counted in the presence of Ultima GoldTM (Perkin Elmer) in a liquid scintillation counter. The kinetic studies shown in Figure 3 and Figures S2-4 were performed using washed pellets, obtained by centrifuging extracts for 20 min at 16 000 x *g*, resuspending them in the initial volume of extraction buffer, recentrifugation and resuspension in the original volume of extraction buffer.

Mutation search

Genomic DNA analysis of the child with hypoacetylaspartia [16] was performed with informed consent of the legal parents. The 3 exons of the NAT8L gene were PCR-amplified using 0.5 μ M of forward and reverse primers (Table S1) with 75 ng of genomic DNA as a template. These reactions (50 μ l) were performed using Biotools DNA polymerase (Kordia Life Sciences, Leiden, the Netherlands) in its provided buffer, 0.2 mM dNTP, and 1 M betaine. PCR products were purified and sequenced directly on both strands by use of a CEQ2000 sequencer (Beckman) or by the sequencing service of Macrogen (Seoul, South Korea). For the screening of the 118 healthy controls, the region surrounding the deletion was amplified from genomic DNA with a specific couple of primers (Table S1). PCR conditions were the same except that a concentration of 1.5 M betaine was used. The PCR products were directly loaded on a 2 % agarose gel containing 0.5 μ g/ml ethidium bromide to distinguish wild-type (223 bp) and mutant (204 bp) alleles.

Neuronal cultures and infection

Primary cultures of cortical neurons were prepared from 17-18 days old Wistar rat embryos as described previously [22]. Brains were dissected, transferred to HBSS buffer medium (1 mM sodium pyruvate, 10 mM Hepes without Ca^{2+} and Mg^{2+}), dissociated in the same medium containing 1.26 mM Ca^{2+} and Mg^{2+} (0.50 mM MgCl_2 and 0.41 mM MgSO_4) and centrifuged for 10 min at 200 g (room temperature). Cells were plated in culture dishes pre-treated with 10 μ g/ml poly-L-lysine in PBS and cultured for 7 days in NEUROBASAL medium supplemented with 2% (v/v) B-27 medium and 0.5 mM L-glutamine prior to infection with recombinant lentivirus. The NAT8L-pEF6MycHisA plasmid was utilized for construction of recombinant lentivirus, as previously described [23]. After 7 days of culture, neurons were incubated in the presence of the lentivirus for 3 days before analysis.

Immunofluorescence in transfected CHO cells and infected primary neurons

CHO cells were propagated in DMEM/F-12 supplemented with 10 % fetal bovine serum (FBS) and antibiotics (100 U/ml penicillin and 100 μ g/ml streptomycin). Cells were seeded at 50,000 cells/cm² in 24-well plates on glass coverslips coated with 25 μ g/ μ l fibronectin (in PBS) and grown for one day (\approx 80 % confluency). Transfection with the NAT8L-pEF6MycHisA plasmid or the PDIb-HA construct [24] was carried out at 0.5 μ g total DNA and 1 μ l lipofectamine/cm² at 37°C for 5 h followed by reincubation overnight in DMEM/F-12/10% FBS without antibiotics. After 24 h, cells were processed for immunofluorescence as described by Mettlen *et al.* [25].

For immunofluorescence experiments, neurons were seeded at 10⁵ cells/cm² on glass coverslips, fixed with paraformaldehyde 4% (v/v) for 15 min at room temperature and permeabilized thereafter with 1% Triton X100 (v/v) in PBS during 15 min. Non-specific immunostaining was prevented by 1 h incubation in a PBS solution containing 3% non-fat dry milk at room temperature.

For primary antibodies, we used mouse IgG_{2a} anti-Myc-Tag (9B11) monoclonal antibody (1/500; Cell Signalling), mouse IgG₁ anti-His monoclonal antibody (1/50; Qiagen), rabbit affinity-purified specific antibodies against NAT8L (1/100), rabbit anti-HA antibodies (1/300), rat monoclonal antibody recognizing KDEL-bearing proteins (1/300; Abcam), either alone or combined with mouse IgG_{2b} monoclonal antibody to PDI (1/50; Abcam), rabbit MAP-2 primary antibody (B9; serum used at 1/1000 dilution) and Alexa-labelled secondary antibodies were from Molecular Probes/Invitrogen (each used at 5 μ g/ml). For live

mitochondria labelling, cells were incubated with MitoTracker-red (CMXRos; Molecular Probes; 250 nM in DMEM/F-12) at 37°C for 30 min followed by chase in MitoTracker-free medium for 30 min, before fixation-permeabilization. All preparations were mounted in Mowiol in the dark overnight and examined with a LSM 510 META confocal microscope (Zeiss, Jena, Germany) using a Plan-Apochromat 63X/1.4 oil DIC objective.

Specific antibodies against NAT8L

Antibodies were raised against two peptides of mouse NAT8L (C)NTAFRGLRQHPRTQLL (P44) and (C)MSVDSRFRGKGIKALG (P45) previously shown to be suitable for immunization [26]. These peptides were conjugated to keyhole limpet hemocyanin and injected together to rabbits 4 times at 2 weeks intervals. Serum was taken from the rabbits one month after the final injection of these peptides. IgG were isolated from the sera by affinity chromatography on protein-G (HITRAP protein G HP, GE Healthcare). IgG were bound to the column in PBS buffer and eluted with 50 mM glycine-HCl, pH 2.7, containing 150 mM NaCl. Collected fractions were immediately neutralized with 1 M Tris-HCl, pH 8. Antibodies specific to the P44 and P45 NAT8L peptides were then purified by affinity chromatography on columns coupled with the P44 or P45 peptide. Peptides were coupled through their amino-terminal cysteine to iodoacetamide immobilized on a crosslinked agarose support (Sulfolink, Thermo scientific PIERCE). IgG fractions were loaded on the peptide-coupled column in PBS. Antibodies bound to the column were eluted with 50 mM glycine-HCl, pH 2.7, containing 150 mM NaCl, and immediately neutralized with 1 M Tris-HCl, pH 8.

RESULTS

Database searches

The characteristics of Asp-NAT are probably shared by only a limited number of mammalian gene products. Indeed, (1) this enzyme most likely belongs to a family of N-acetyltransferases; (2) it is mainly, if not exclusively, expressed in brain; (3) it is membrane-bound; (4) it should have close homologues only in species that synthesize NAA. We decided therefore to search mammalian genomes for genes encoding proteins with these characteristics.

A search in the human and mouse genomes with N-acetyltransferase (NAT) as a query yielded about 30 genes encoding N-acetyltransferases. These comprised a few well-identified sequences (e.g. N-acetyltransferases acting on arylamines, glucosamine-6-phosphate, glutamate, and spermine/spermidine) and a majority of putative N-acetyltransferases (i.e. hypothetical proteins showing sequence similarity to well characterized N-acetyltransferases) with undefined specificity. mRNA expression data of the SymAtlas database [27] indicated that two of them were mainly present in brain: NAT14 and NAT8L. NAT14, also called Klp1 (K562 cell-derived leucine-zipper-like protein 1) is thought to be a transcription factor regulating the expression of coproporphyrinogen oxidase by binding to a promoter regulatory element [28]. NAT8L, also designated Shati, is overexpressed in brain after administration of metamphetamine to mice [26]. Both proteins showed at least one putative transmembrane domain. Close homologues (> 50 % sequence identity) of NAT8L and NAT14 were only found in vertebrates. Taken together, these findings indicated that both NAT8L and NAT14 were good candidates for Asp-NAT.

Gene structure, tissue distribution, and protein sequence analysis of NAT8L

NAT8L is encoded by 3-exon genes on mouse chromosome 5 and human chromosome 4 (band 4p16.3). Previous PCR data obtained with mouse NAT8L (Shati) indicated that the mRNA encoding this protein is expressed not only in brain, but also in liver and kidneys [26]. We therefore performed quantitative PCR amplification (as described in [18]) on mouse tissue cDNA with couples of primers situated in exons 1 and 2, or in exons 2 and 3. Our findings indicated that NAT8L was amplified from brain cDNA and to a lesser extent from thymus or spleen cDNA. The abundance of the mRNA was calculated to be about ten-fold lower in these tissues than in brain. No specific amplification was observed with liver, kidney, lung, skeletal muscle, testis, and heart cDNA (results not shown).

The NAT8L cDNA was also amplified from human brain cDNA using primers situated in exons 1 and 3. Sequencing of the amplified products confirmed the deduced protein sequence registered under NCBI accession number EAW82541. No amplification could be observed with primers corresponding to a cDNA sequence encoding a protein (Swissprot Q8N9F0) that differs from human and mouse NAT8L in its first \approx 90 residues. This cDNA contains in fact an exon from the upstream human gene (LOC401115 in human) fused to exons 2 and 3 of the NAT8L gene.

The NAT8L sequences of various species are aligned in Figure 1. As previously noted [26], the C-terminal region is homologous to N-acetyltransferases. NAT8Ls also comprise a conserved N-terminal sequence of 15 residues, a proline- and alanine-rich region of variable length (residues 40-73 in the human sequence), and a highly hydrophobic region of about 30 amino acids that just precedes the N-acetyltransferase domain.

Targeting prediction programmes (TargetP [29]; PSort2 [30]) did not suggest that NAT8L had a mitochondrial propeptide or a signal peptide. It should be noted that exon 1 of

human, mouse and rat NAT8L genes contains an in-frame stop codon that precedes the initiator ATG codon. This indicates that the protein sequence truly starts with the methionine shown as Met1 in the alignment. NAT14 is not described in detail as we show in the next section that it does not correspond to Asp-NAT.

Identification of aspartate N-acetyltransferase as NAT8L

Both mouse NAT8L and NAT14 were overexpressed in HEK293T cells, as native proteins or as fusion proteins with a polyHis tag at the C- or the N-terminus. GC-MS analysis [21] of media collected after 48 hours indicated the presence of a product co-eluting with authentic NAA in medium from cells transfected with all NAT8L constructs (see Figure S1 in the Supplementary data), whereas this compound was below the detection limit (5 μM) in control cells (Figure S1A), or those transfected with NAT14 constructs. The fragmentation pattern of this product (insert of Figure S1B) was identical with that of authentic NAA, with major fragments of m/z ratios 73, 147, 184, 245, 274 and 376 [21]. Quantification of NAA by isotope dilution with the trideuterated compound (Figure 2B) indicated that the concentration of NAA in the medium amounted to 200-250 μM in cells transfected with the different NAT8L constructs, corresponding to an amount of ≈ 300 nmol/mg cell protein. Analysis of cell extracts indicated that this compound was also present intracellularly at concentrations of about 60 nmol/mg protein (results not shown).

Asp-NAT activity was assayed in cell extracts as the acetyl-CoA-dependent conversion of [^{14}C]L-aspartate to a more acidic product (Figure 2C). In full agreement with the results mentioned above, this activity was observed in extracts of cells transfected with the three types of NAT8L constructs, but not in other extracts. Slightly lower activities were observed with the N-tagged and the C-tagged forms as compared to untagged NAT8L (59 % and 75 %, respectively). Western blot analysis with an anti-polyHis tag antibody (Figure 2A) revealed that all His-tagged versions of NAT8L and NAT14 were expressed. This indicated that the absence of Asp-NAT activity in cells transfected with NAT14 was not due to lack of expression of this protein.

Kinetic properties of NAT8L

The Asp-NAT activity was further characterized in extracts of cells expressing wild-type mouse NAT8L. The enzymatic activity was almost completely ($> 90\%$) recovered in the pellet after a 20-min centrifugation of frozen-thawed extracts at $16.000 \times g$ (not shown), indicating that it is membrane-bound. The kinetic studies were therefore performed on washed pellets, to eliminate soluble, potentially interfering enzymes. Detergents showed a biphasic effect on the activity (Figure 3A), increasing it at low concentration, presumably because they allowed access of the catalytic site to substrate, and being inhibitory at higher concentrations. Double-reciprocal plots of the saturation curve for aspartate was linear both in the absence and in the presence of 0.5 mM CHAPS, which increased V_{max} by $\approx 30\%$ without affecting the K_{M} ($\approx 90 \mu\text{M}$) for aspartate (Figure S2). With acetyl-CoA as varying substrate, the double-reciprocal plots were non-linear in the absence of detergent, consistent with part of the enzyme being poorly accessible to acetyl-CoA, but well in the presence of CHAPS (Figure 3B). The K_{M} values amounted to ≈ 9 and $13 \mu\text{M}$ in the presence of 0.5 and 1 mM CHAPS, respectively.

[^{14}C]Glutamate was also used by the enzyme, but with an about 50-fold lower affinity ($K_{\text{M}} \approx 5 \text{ mM}$) and a similar V_{max} compared to aspartate (not shown), in agreement with previous results [31,32]. Accordingly, the incorporation of [^{14}C]-aspartate into NAA was inhibited by glutamate competitively with respect to aspartate with a K_{i} of $\approx 6 \text{ mM}$ (Figures

3C and S3A). No inhibition was observed with other amino acids, indicating that the enzyme is specific. The enzyme was also inhibited by its reaction product NAA. This inhibition was essentially competitive with respect to aspartate ($K_i = 0.56$ mM; Figures 3D and S3B) and uncompetitive versus acetyl-CoA (Figure S4).

Subcellular localization of NAT8L

Because of the controversy on the subcellular localization of Asp-NAT, it was of interest to check the localization of NAT8L in transfected cells. This was done by confocal microscopy analysis of CHO cells transfected with *myc*-tagged (at the C-terminus), *his*-tagged (at the N-terminus) or untagged NAT8L, and of neurons in primary culture infected with a lentiviral vector allowing the expression of *myc*-tagged NAT8L. Immunolabelling of *myc*-tagged NAT8L expressed in CHO cells or in primary neurons delineated the nuclear envelope (arrows in Figures 4 and 5) and produced a reticular pattern, strongly suggestive of the endoplasmic reticulum. This was confirmed by the substantial (though not complete) co-localization of *myc*-tagged NAT8L with KDEL-bearing proteins of the endoplasmic reticulum (arrowheads in Figures 4 and 5). Analysis of the colour intensity profiles in the cytoplasm showed that the peaks of NAT8L-labelling most often coincided with the peaks of ER marker labelling (Figures 4D and 5C; see also Figure S5C). Large but not full co-localization is in keeping with the well-known heterogeneity among endoplasmic reticulum domains [33,34]. In contrast, the *myc*-tagged NAT8L signal was fully resolved from the Golgi complex, recognized by co-transfection with the PD1b-HA construct [24] (Figure 4B), and from mitochondria labelled *in vivo* by MitoTracker red (Figures 4C, 4E), as confirmed by analysis of the intensity profiles (Figure 5B). Targeting to the nuclear envelope and the endoplasmic reticulum was independent of epitope tagging, since NAT8L fused to a *myc*-tag at its C-terminus or to a polyHis-tag at its N-terminus showed a similar localization pattern to native NAT8L recognized with affinity-purified antibodies (Figure S5A).

Mutation of NAT8L in a patient with hypoacetylaspartia

To check whether NAT8L was mutated in the patient deficient in NAA, we amplified the three exons of the NAT8L gene and screened them for mutations. A 19-bp deletion in the homozygous form was observed in the first exon (Figure 6A). This mutation leads to a change in the reading frame after amino acid 69 and it should therefore lead to no production of functional protein. To rule out the possibility that this mutation would still allow the production of an active protein by codon-slipping or by reinitiation at a later AUG codon, we prepared an expression vector for this mutated form (provided with a C-terminal his-tag) and transfected it in HEK293T cells. Extracts prepared from these cells did not contain any detectable Asp-NAT activity or material reacting with the anti-polyHis tag antibody, whereas extracts of cells transfected with the non-mutated human cDNA were enzymatically active and contained an immunoreactive band of the appropriate size (Figure 6B and C).

Several human NAT8L protein entries (e.g. BAC04426.1) comprise only the last 134 AA of NAT8L. The sequence of this (putative) shorter protein is unaffected by the mutation and it was therefore important to verify if it displayed Asp-NAT activity. Transfection of a vector allowing the expression of this shorter form resulted in the expression of a polypeptide of the expected size (≈ 18 kDa) but to no detectable enzymatic activity (Figure 6B and C). Taken together, these findings indicated that the mutation found in the patient corresponds indeed to a null mutation.

The patient is an adopted child and no material was therefore available to check the presence of the mutation in the biological parents. The mutation found in the patient could be

detected by PCR amplification of genomic DNA and gel electrophoresis. This assay allowed also the detection of the mutation in the heterozygous state, as indicated by amplifications performed on samples containing equal amounts of DNA from the patient and a control. The 19-bp deletion was not found in 118 control DNA samples either in the homozygous or the heterozygous state (results not shown).

DISCUSSION

Molecular identification of aspartate N-acetyltransferase

Despite its discovery about 50 years ago [2], Asp-NAT had not yet been molecularly identified, owing to the difficulty of purifying this membrane-bound enzyme [7]. The availability of databases comprising virtually all proteins encoded by the human and mouse genomes and providing information on their tissue distribution enabled us to identify two candidate proteins for this enzyme. Transfection studies in HEK293T cells showed that NAT8L-expressing cells synthesize NAA and that extracts of these cells display Asp-NAT activity. This activity had properties similar to those of brain Asp-NAT, including its K_M value for aspartate, its specificity for this amino acid, its inhibition by the reaction product, its association with membranes and its inhibition by detergents [4,5,7,32]. These findings therefore indicate that NAT8L is responsible for NAA synthesis in brain. This conclusion is strongly supported by the finding that the NAT8L gene is mutated in the only patient reported to have undetectable levels of NAA in brain. Considering that NAA is restricted to neurons, where it is synthesized, our identification is also consistent with immunohistochemical studies showing that mouse shati (i.e. NAT8L) is exclusively present in neurons [26].

As expected for Asp-NAT, close homologues of NAT8L were found in vertebrates, whose brain contains NAA, but not in insects and *Caenorhabditis elegans*, where NAA has never been reported. Unfortunately, the genome of the crayfish, in which NAA has been described [35], has not yet been sequenced.

The molecular basis of hypoacetylaspartia

The finding that the NAT8L gene is mutated in the single known case of hypoacetylaspartia [15-17] indicates that this disorder, in which no NAA is detectable in brain, is due to a lack of synthesis of this compound in brain. This excludes other potential causes, such as an increased degradation by a hyperactive aspartoacylase, or a deficiency in the transport or the formation of aspartate and/or acetyl-CoA in the compartment in which aspartate N-acetyltransferase is present. The finding that the mutation induces a change in reading frame indicates that it is a *null* mutation. Accordingly, no NAA synthase activity could be detected in cells that had been transfected with a plasmid carrying the mutated allele.

The homozygous mutation found in the patient suggests that he may be the offspring of consanguineous parents. Unfortunately, no information is available on the biological parents of this adopted child. Therefore one has to be very cautious before concluding that the phenotype of this unique patient is representative of hypoacetylaspartia, as he may suffer from other recessive genetic diseases.

It is likely that hypoacetylaspartia is underdiagnosed, and this for two reasons. Firstly, MRS is not systematically performed to investigate neurological problems in young children. Secondly, decreased levels of NAA are usually interpreted as being *secondary* to a loss of neuronal integrity, rather than as a *primary* cause of neuronal dysfunction. However, point mutations in the NAT8L gene that would not lead to a complete loss of enzymatic activity could result in low, yet detectable levels of NAA and be associated with milder forms of the

disease than the one described in the only patient known so far. It would therefore be of interest to determine more systematically the brain NAA level in young patients with mental retardation, epilepsy of unknown origin, and/or ataxia, and to search the NAT8L gene for mutations if the NAA level is found to be decreased.

Subcellular localization of aspartate N-acetyltransferase

As mentioned in the introduction, the subcellular localization of Asp-NAT is still a matter of controversy. Fractionation studies of brain extracts have variously concluded to a microsomal [4] or a mitochondrial localization of Asp-NAT [6,7]. However, one of the studies concluding to a mitochondrial localization shows in fact that the specific activity of Asp-NAT is higher in the microsomal than in the mitochondrial fraction [7], which in fact argues for a microsomal localization. The finding [6] that purified brain mitochondria produce NAA when incubated with glutamate (a source of aspartate) and pyruvate (sources of acetyl-CoA in the mitochondrial matrix) is taken as evidence that intact brain mitochondria contain Asp-NAT. One has to note, however, that the assay used by Patel and Clark [6] for measuring the NAA produced by mitochondria employed a rather crude (7-fold purified) preparation of pig kidney aspartoacylase to convert NAA to aspartate. Therefore, another metabolite (asparagine, for instance) may have been measured. These contradictory findings are most likely the result of the inherent difficulty of studying the subcellular distribution by fractionation in such a heterogeneous tissue as brain.

Recent tracer studies comparing the incorporation of radioactivity into NAA from L-aspartate and L-malate in SH-SY5Y neuroblastoma cells have led Arun *et al.* [36] to conclude that mitochondria are a major site of NAA synthesis. However, the technique used (thin layer chromatography) to isolate radiolabelled NAA and L-aspartate does not allow one to separate these metabolites from other radioactive metabolites that certainly form when cells are incubated for 3 hours with a radioactive precursor, making that the results of this work have to be interpreted with caution.

Low levels of NAA were found in the brains of mice deficient in ARALAR [37], a glutamate/aspartate exchanger serving to export aspartate from brain mitochondria. This was taken to suggest that aspartate has to be transferred from the mitochondria to the cytosol before being acetylated by aspartate N-acetyltransferase. This argument, though pleading against a mitochondrial localization of Asp-NAT, is not decisive because one may not exclude that a defect in ARALAR also causes a decrease in the mitochondrial concentration of aspartate.

Our own data on the localization of NAT8L (i.e., Asp-NAT) indicate that this protein does not co-localize with mitochondria, consistent with the absence of a mitochondrial propeptide in the NAT8L sequence. The reticular pattern of the expression with labelling of the nuclear membrane and the (at least partial) co-localization with endoplasmic reticulum markers, indicate instead that NAT8L is associated with the endoplasmic reticulum. This localization is independent from the tag used for immunostaining and is observed not only in CHO cells, but also in neurons in primary cultures. These findings lead therefore to the conclusion that Asp-NAT is associated with the endoplasmic reticulum.

Perspectives

Our work opens the possibility of developing experimental models to better define the role of NAA. None of the roles proposed so far accounts for the very high concentration of NAA in neurons, since NAA does not appear to be a neurotransmitter [13] and since its role as an osmolyte is at best very minor compared to that of other solutes present in neural cells [38].

The role of NAA in myelination may even be questioned since this process is only moderately delayed in the patient with NAA deficiency [16-17]. The creation of a mouse knock-out model may also permit to determine to what extent the symptoms found in the patient with hypacetyaspartia are due to NAA deficiency. The availability of the Asp-NAT sequence will also allow for a better definition of the mechanisms that control the concentration of this compound, including the effect of pathological conditions [39] and antipsychotic drugs [40]. In this respect, the finding that Shati (i.e. NAT8L) is upregulated following metamphetamine treatment suggests that NAA plays a role, presumably an indirect one, in dopaminergic transmission [26,41]). This work confirms also the need to perform MRS spectroscopy in children with developmental problems, especially when microcephaly is present, to check for NAA deficiency.

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FIGURE LEGENDS

Figure 1. Multiple alignment of NAT8L sequences. The alignment shows the sequences from *Homo sapiens* (Hsap; NCBI accession nr EAW82541), *Mus musculus* (Mmus; NP_001001985) and *Danio rerio* (Drer; NP_001077308). Strictly conserved residues in the three sequences are indicated in bold. A 30 residue hydrophobic region is underlined in the human sequence.

Figure 2. Effect of transfection of HEK cells with mouse NAT8L or NAT14 on the NAA concentration in the medium and the Asp-NAT activity in cell extracts.

Cells were transfected with plasmids expressing NAT8L or NAT14, either as such (WT) or as C-terminal (CT) or N-terminal (NT) polyHis tag fusion proteins. Transfection with an empty plasmid (EP) was used as a control. After 48 h, the NAA concentration was measured by GC/MS in the medium (B) and the Asp-NAT activity was assayed in cell extracts (C). The indicated extracts were also analysed by Western blotting with an anti-polyHis tag antibody to evaluate the level of expression of the recombinant proteins (A). These data are representative (A) or the means \pm SEM (B, C) of three independent transfection experiments.

Figure 3. Kinetic properties of the Asp-NAT activity of NAT8L.

HEK cells were transfected with untagged mouse NAT8L and the enzymatic activity was determined radiochemically on washed membrane pellets as described in the Materials and Methods. (A) Effect of two detergents on the Asp-NAT activity; assay medium contained 50 μ M L-aspartate, 200 μ M acetyl-CoA, and the indicated concentrations of CHAPS or octylglucoside. (B) Effect of CHAPS on the saturation curve for acetyl-CoA; assay medium contained 20 μ M aspartate and the indicated concentrations of acetyl-CoA and CHAPS. (C) Effect of glutamate and (D) of N-acetylaspartate on the saturation curve for aspartate; assay medium contained 200 μ M acetyl-CoA, 0.5 mM CHAPS and variable concentrations of aspartate. Panels (B), (C) and (D) show double reciprocal plots. The results shown are the means \pm SEM for three determinations performed with extracts derived from independent transfections. In some cases, the SEM is not visible because it is smaller than the size of the symbol.

Figure 4. Co-localization of *myc*-tagged NAT8L with the endoplasmic reticulum (ER) and complete segregation from the Golgi complex and mitochondria (mito).

CHO cells were transfected to express low levels of C-terminal *myc*-tagged NAT8L and immunolabelled with an antibody against the *myc*-antigen (photographs of the central column; green in 'merge' photographs). (A) Double immunolabelling for KDEL-bearing proteins to label the ER (left photograph; red in 'merge'). (B) Cells were co-transfected with the PD1b-HA construct to immunolabel the Golgi apparatus (left; red in 'merge'). (C) Transfected cells were incubated with MitoTracker red before fixation (left; red in 'merge'). Notice the nuclear envelope labelling by NAT8L (arrows in the middle photographs) and its reticular pattern in the cytoplasm with substantial co-localization with the ER marker (arrowheads), contrasting with full segregation from Golgi and mitochondrial labelling. Scale bars, 5 μ m. (D, E) Intensity profiles along the paths indicated by the arrows in the right-hand photographs of panels A and C, respectively.

Figure 5: Co-localization of *myc*-tagged NAT8L with ER and its segregation from mitochondria in neurons. Primary cultures of rat fetal neurons were infected with lentiviruses encoding C-terminal *myc*-tagged NAT8L (see SI Text), fixed at a low level of expression, and immunolabelled for *myc* antigen (green in 'merge'). (A) Triple

immunolabelling with ER markers (KDEL combined with PDI antibodies, red in 'merge') and MAP-2 to establish neuronal identity (blue). (B) Neurons were incubated with MitoTracker (red), then immunolabelled for *myc* antigen (green in 'merge') and MAP-2 (blue). The complete delineation of the nuclear envelope by NAT8L is best seen in the upper panel (arrows), which also evidences the same reticular pattern as for ER markers. In neurons, MitoTracker is known to produce much weaker signals than in CHO cells, yet it is also clearly segregated from NAT8L. Scale bars, 5 μ m. (C) Intensity profile along the path indicated by the arrow in the 'merge' photograph of panel A.

Figure 6. Mutation found in the patient with hypoacetylaspartia and its effect on the activity and expression of NAT8L

(A) Sequences of nucleotides 190-240 of the NAT8L open reading frame and deduced protein sequences in controls and in the patient. Nucleotide 1 corresponds to the adenine of the initiator ATG codon. The 19 nucleotides deleted in the patient are shown in small characters and the 11 bp repeats between which this deletion occurred is underlined. (B and C) HEK293T cells were transfected with an empty plasmid (EP) or a plasmid driving the expression of human wild-type NAT8L (Met1), the form with the 19-bp deletion (del19), and a shorter form corresponding to amino acids 169-302 (Met169). All proteins contained a C-terminal polyHis tag. (B) Asp-NAT activity in cell extracts. (C) Western blots with an anti-polyHis tag antibody.

Hsap **MHCGPPDMVCETKIVA**AED**HEALPGAKKD**ALLAAAGAMWPPL**PAAPGPAAAP** 52
 Mmus **MHCGPPDMVCETKIVA**TED**HEALPGAKKD**ALLVAAGAMWPPL**PAAPGPAAAP** 52
 Drer **MHCSSPKMVCETKIVA**--DE**HEA**IAGTKKDSIIVSSSQ**MWTSSSASP**----- 45

Hsap PAPPAPVAQPHGGAGGAGPPGGRG**VCIREFRAAEQEAARRIFYDGIMERIP** 104
 Mmus ---PPAAGPQPHGGTGGAGPPEGRG**VCIREFRAAEQEAARRIFYDGILERIP** 101
 Drer -----SALESKIEKRNQ**VFIREFERSDHEEVRRIFNEGIMERIP** 84

Hsap **NTAFRGLRQH**PRA**QLLYALLAALCF**AVSR**SLLLTCLVPAALLGLRYYYYSRKV** 156
 Mmus **NTAFRGLRQH**PR**TQLLYALLAALCF**AVTR**SLLLTCLVPAGLLALRYYYYSRKV** 153
 Drer **NSAFRGLKQ**QTT**QFMYAFL**TVMCYVMT**KSFTLTFCAPFILMGARYYYYYSRKV** 136

Hsap **IRAYLECALHTDMADIEQYYMKPPGSCFWAVLDGNVVGIVAAARAHEEDNTV** 208
 Mmus **ILAYLECALHTDMADIEQYYMKPPGSCFWAVLDGNVVGIVAAARAHEEDNTV** 205
 Drer **ILSYLDCALHTDMADIEAYYMKPTGSCFWAVLQGGVVGIVAAQSR**EDDNTV 188

Hsap **ELLRMSVDSRFRGKGI**AKALGRK**VLEF**AVVHNYS**SAVVLGTTAVKVA**AAHKLYE 260
 Mmus **ELLRMSVDSRFRGKSI**AKALGR**RVLEF**AMLHNYS**SAVVLGTTAVKVA**AAHKLYE 257
 Drer **ELRRMSVDSHFRGKGI**AKALGR**RVIEF**AMLNNYS**SAVVLGTTAVKMA**AAHKLYE 240

Hsap **SLGFRHMGASDHVLPGMTLSLAERLFFQVRYHRYRLQLEE** 302
 Mmus **SLGFRHMGASDHVLPGMTLSLAERLFFQVRYHRYRLQLEE** 299
 Drer **SLGFRRVGETEDYTLPGMTRSPLERLFFQIRYSHYRLQHEE** 282

Figure 1

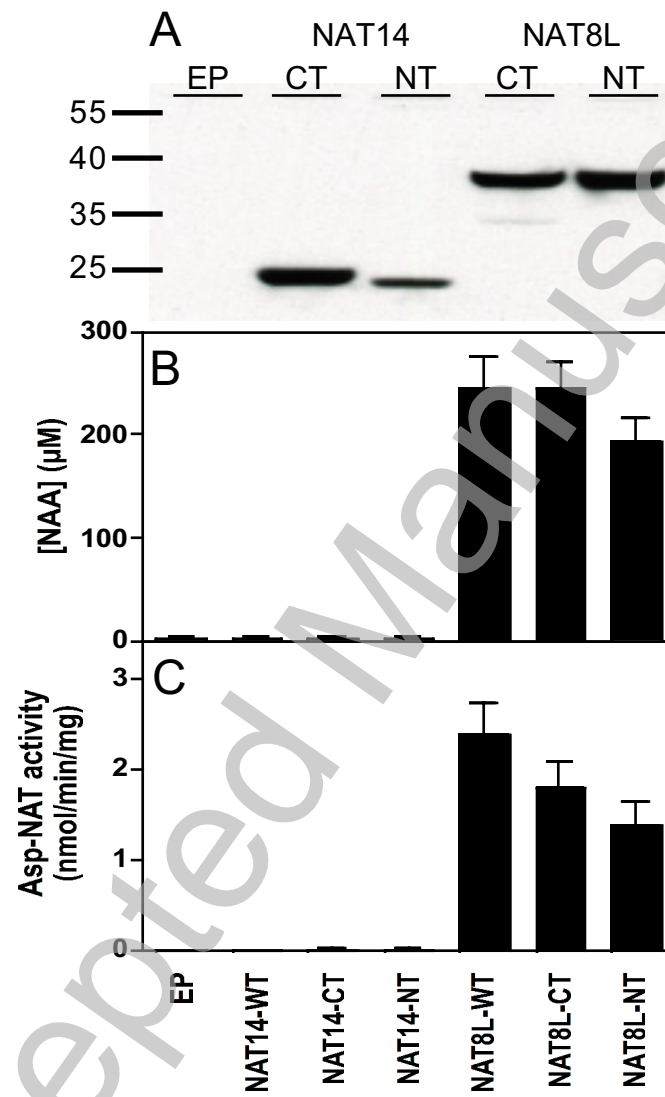


Figure 2

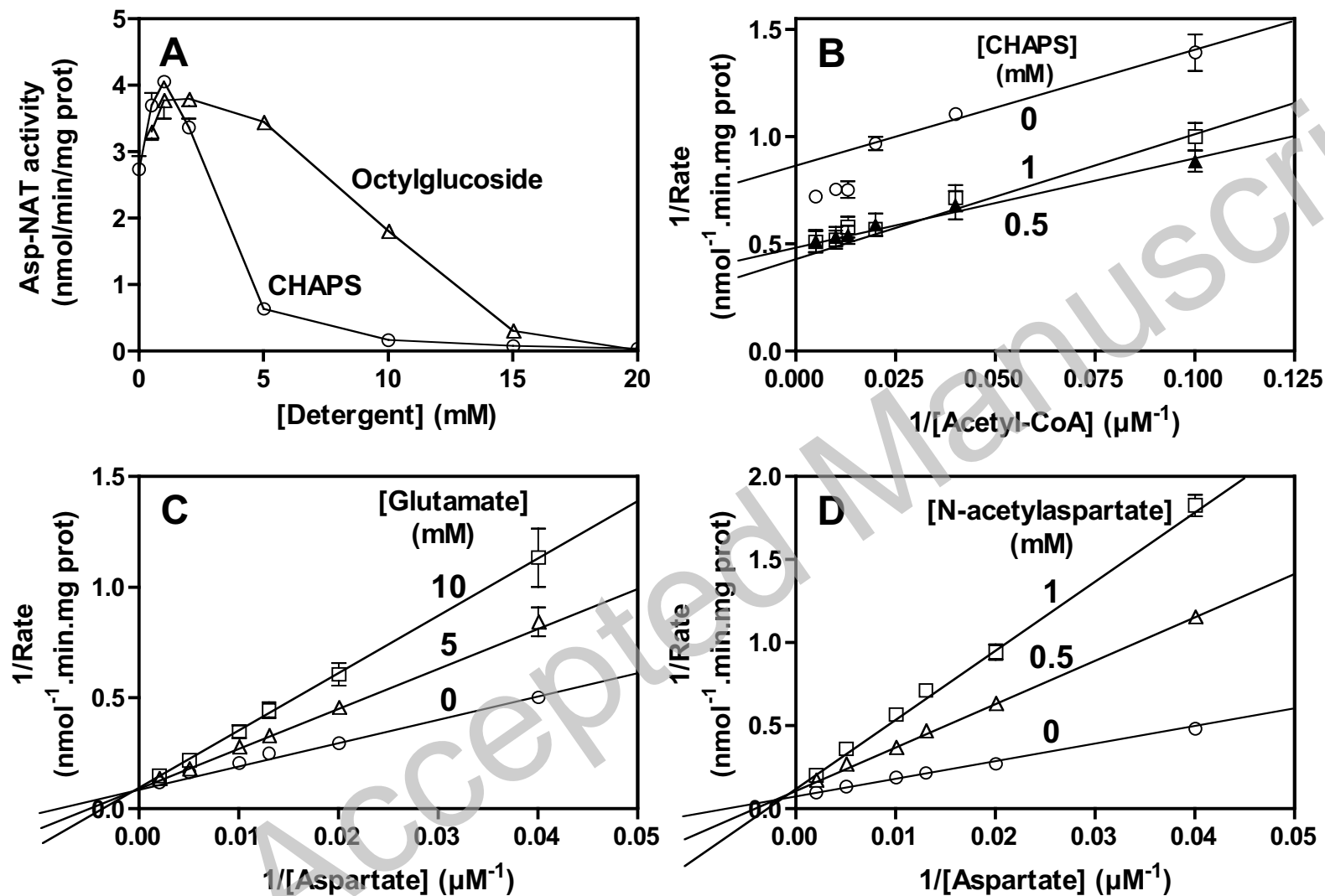


Figure 3

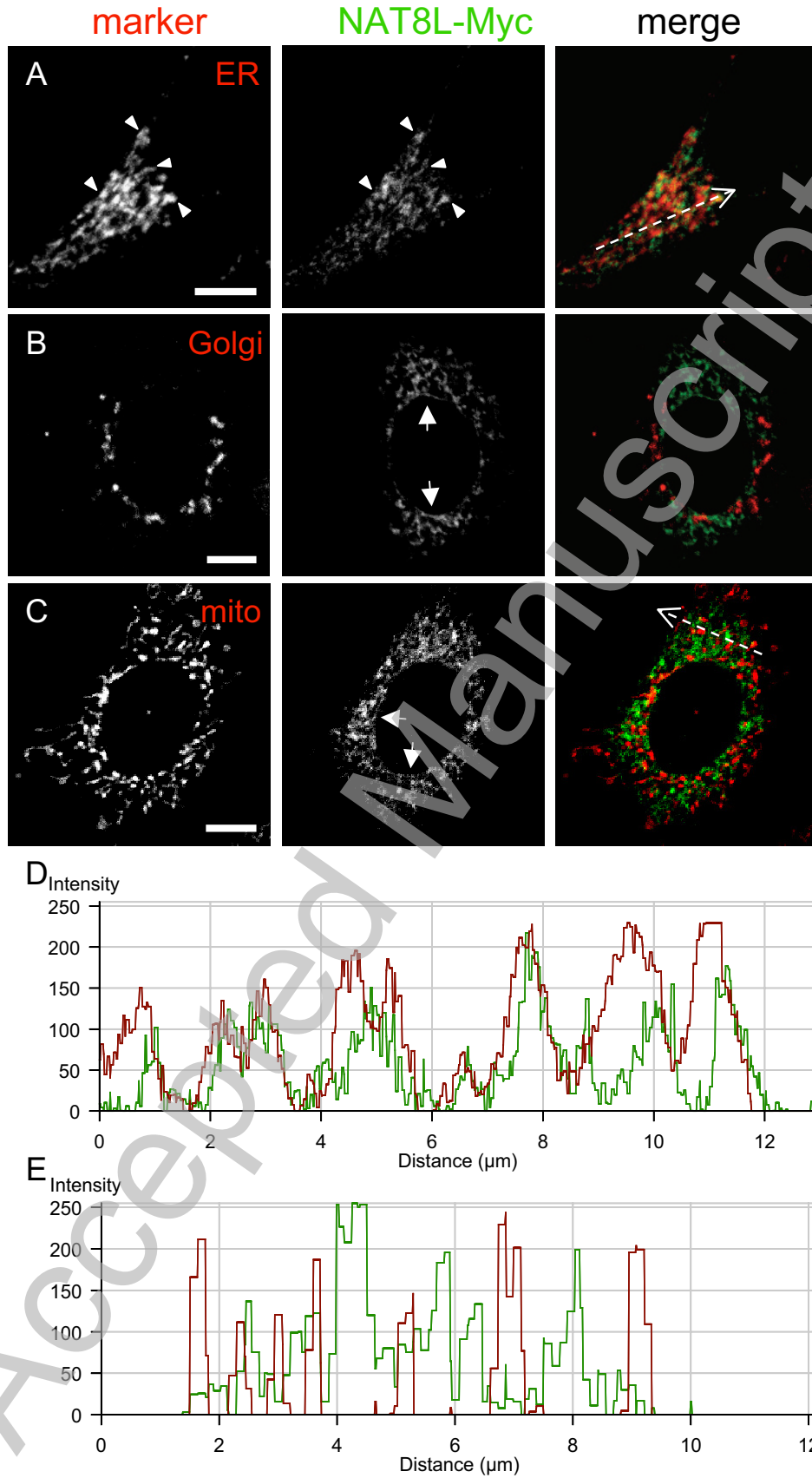


Figure 4

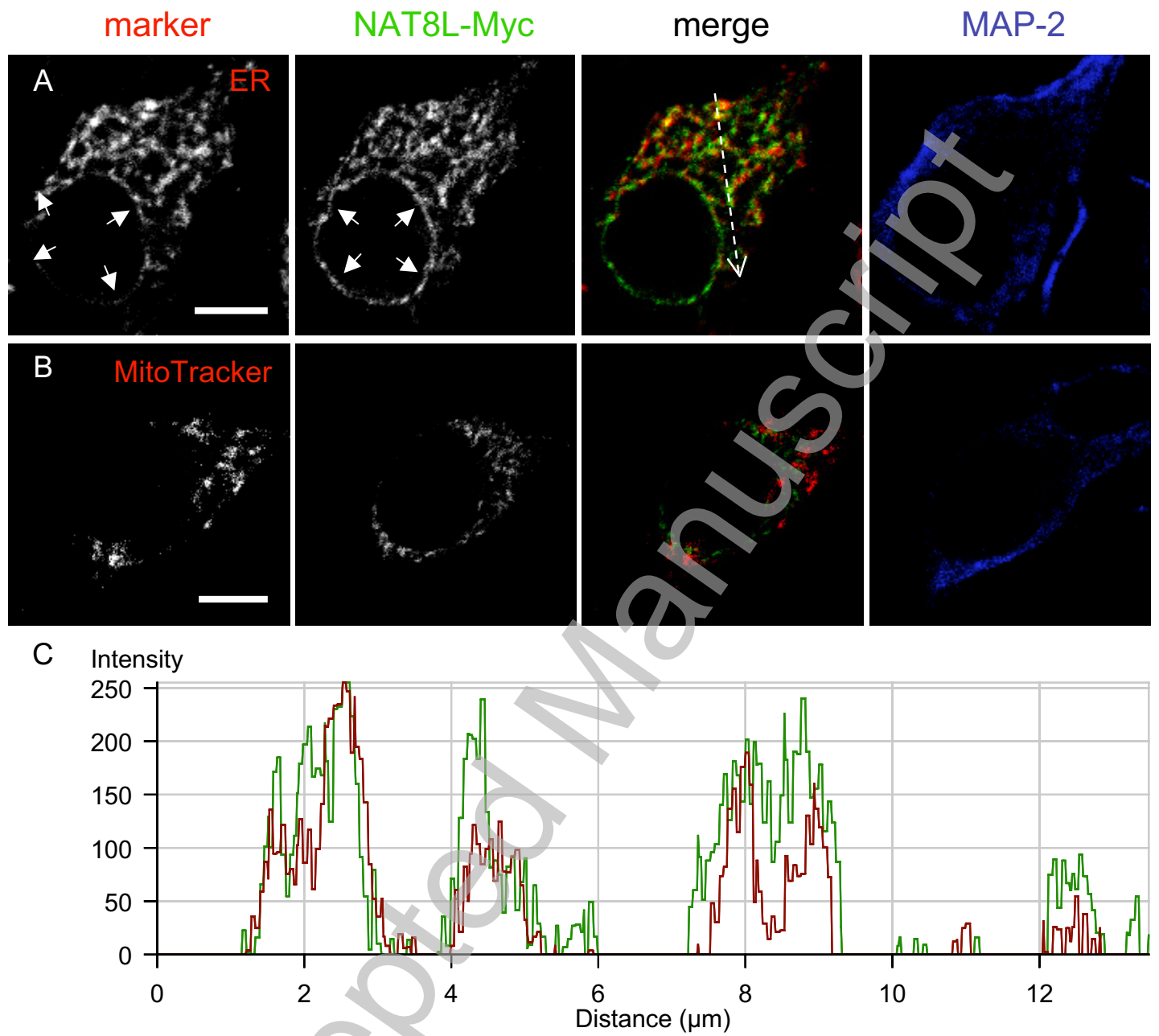


Figure 5

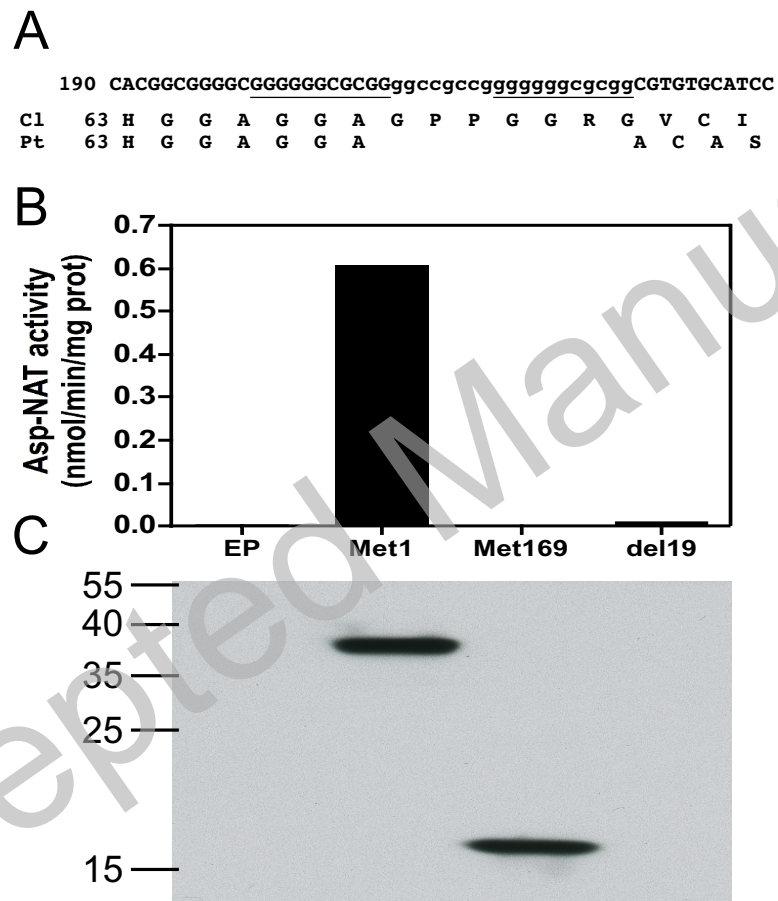


Figure 6