学位論文

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A quantitative study on splice variants of *N*-acylethanolamine acid amidase in human prostate cancer cells and other cells

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ABSTRACT

N-Acylethanolamine acid amidase (NAAA) is a lysosomal enzyme, hydrolyzing various bioactive *N*-acylethanolamines with a preference for palmitoylethanolamide. Human NAAA mRNA was previously reported to consist of multiple 3′-end splice variants. However, their tissue distributions and roles have not been examined yet. In the present study, we first identified four major splice variants (tentatively referred to as a1, a2, b2, and c2) in a human prostate cancer cell line LNCaP, which were composed of exons 1–11, exons 1–10 and 12, exons 1–9 and 12, and exons 1–8 and 12, respectively. We next developed quantitative polymerase chain reaction methods to individually quantify these NAAA variants as well as collectively measure all the variants. Among various human prostate cancer cells, the total levels of NAAA mRNAs in androgen-sensitive cells like LNCaP were higher than those in androgen-insensitive cells. In all of these prostate cells and other human cells, variants a1 and b2 showed the highest and lowest expression levels, respectively, among the four variants. Interestingly, ratios of the four variants were different by cell type. Variants a1 and a2 encoded the same full-length NAAA protein, which was catalytically active, while b2 and c2 were translated to C-terminally truncated proteins. As expressed in HEK293 cells these truncated forms were detected as catalytically inactive precursor proteins, but not as mature forms. These results revealed wide distribution of multiple variants of NAAA mRNA in various human cells and suggested that the proteins from some variants are catalytically inactive.

1. Introduction

N-Acylethanolamines are ethanolamides of long-chain fatty acids and ubiquitously exist in animal tissues [1,2]. *N*-Acylethanolamines are well known as a class of endogenous lipid mediators, showing different biological activities depending on acyl species. Arachidonoylethanolamide (anandamide) has been most extensively studied as an endogenous ligand of cannabinoid receptor CB1, which is thus defined as an endocannabinoid [3], as well as an endogenous ligand of transient receptor potential vanilloid 1 (an endovanilloid) [4]. In addition to marijuana-like pharmacological activities, endocannabinoids, including anandamide, may be involved in the growth and transformation of tumor cells [5–7]. On the other hand, cannabinoid receptor-insensitive palmitoylethanolamide and oleoylethanolamide bind to other receptors such as peroxisome proliferator-activated receptor-α (PPAR-α) [8,9] and show anti-inflammatory, analgesic, appetite-suppressing, and anti-obesity effects [10,11].

Fatty acid amide hydrolase (FAAH) [12] and *N*-acylethanolamine acid amidase (*N*-acylethanolamine-hydrolyzing acid amidase, NAAA) [13–15] are two major enzymes responsible for the degradation of *N*-acylethanolamines in animal tissues. FAAH is a membrane-associated enzyme with an optimal pH value of 8.5–10, while NAAA is a lysosomal enzyme acting at acidic pH [16,17]. NAAA hydrolyzes various *N*-acylethanolamines with a preference for palmitoylethanolamide [14], and its specific inhibitors increase endogenous levels of palmitoylethanolamide and other *N*-acylethanolamines [18–20]. Specific NAAA inhibitors are thus expected as anti-inflammatory and analgesic drugs. NAAA is distributed in various tissues with predominant expression in macrophages [21,22] and prostate [23]. Recently, Liu et al. reported that NAAA is abundantly expressed in non-aggressive prostate cancer and is potentially useful as a tissue biomarker related to cancer aggressiveness [24].

Hong et al. showed that human *NAAA* gene is composed of 11 exons and that the full length mRNA can be translated to a protein composed of 359 amino acid residues (GenBank™ accession number NM_014435.3) [25]. However, Goodchild et al. had earlier reported that human placenta expresses multiple splice variants of NAAA and that one of them contains exon 12 instead of exon 11 [26]. In agreement

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with this report, many splice variants are found for human NAAA on NCBI database. All the splice variants contain exons 1–8 in common, and alternative splicing occurs in exons 9–12. However, the tissue distribution of each variant and the catalytic activity of the proteins translated have not been investigated. Considering a potential usefulness of NAAA variants as tissue biomarkers, it was also interesting to elucidate how the percentage composition of NAAA variants is different among various cells, which differ in characteristics and origins.

In the present study, we first focused on six splice variants (NM_014435.3, XM_005262923.2, XM_005262924.2, XM_005262920.2, XM_006714180.2, and NM_001042402.1) to which we tentatively refer as a1, b1, c1, a2, b2, and c2, respectively (Fig. 1). a1, b1, and c1 form a group of variants containing exon 11, while a2, b2, and c2 contain exon 12. Since a1, a2, b2, and c2 were found to be major NAAA variants in the prostate cancer cell line LNCaP, we investigated how these four splice variants of NAAA are differently expressed in various cells originated from human prostate and other tissues. We also expressed recombinant NAAA protein isoforms corresponding to these variants as well as several related mutant proteins in mammalian cells and examined their enzyme activities.

2. Materials and methods

2.1. Materials

RPMI 1640 medium, Dulbecco's modified Eagle's medium (DMEM), DMEM/Ham's F-12, Tween 20, and dithiothreitol (DTT) were purchased from Wako Pure Chemical (Osaka, Japan); fetal calf serum (FCS) was from Biowest (Nuaillé, France); phenol red-free RPMI 1640 medium, non-essential amino acid solution, charcoal stripped FCS, Moloney murine leukemia virus reverse transcriptase, pcDNA3.1(+) and pCR4 Blunt-TOPO vectors, and Lipofectamine 2000 were from Invitrogen/Thermo Fisher Scientific (Carlsbad, CA, USA); QuikChange site-directed mutagenesis kit was from Agilent Technologies (Santa Clara, CA, USA); human prostate RNA was from Clontech/Takara Bio (Mountain View, CA, USA); Pierce Western Blotting Substrate Plus was from Thermo Fisher Scientific (Waltham, MA, USA); LNCaP, DU145, and PC3 cells were from the RIKEN BRC Cell Bank (Tsukuba, Japan); THP-1, MCF-7, and human embryonic kidney 293 (HEK293) cells were from Health Science Research Resources Bank (Osaka, Japan); Clonetics Prostate Epithelial Cell Systems, containing human prostate epithelial cells (PrEC) and Prostate Epithelial Cell Basal Medium, were from Lonza (Walkersville, MD, USA); Ex Taq DNA polymerase, random hexamer, PrimeSTAR HS DNA polymerase, PrimeScript RT reagent kit, and SYBR Premix Ex Taq II were from Takara Bio (Ohtsu, Japan); RNeasy mini kit was from Qiagen (Hilden, Germany); palmitoylethanolamide was from Cayman Chemical (Ann Arbor, MI, USA); [1-^{14}C]palmitic acid was from PerkinElmer Life Sciences (Boston, MA, USA); precoated silica gel 60 F_{254} aluminum sheets for thin-layer chromatography (TLC) (20 × 20 cm, 0.2-mm thickness) and Immobilon-P were from Merck Millipore (Darmstadt, Germany); protein assay dye reagent concentrate was from Bio-Rad (Hercules, CA, USA); 100 bp DNA Ladder and peptide-*N*-glycosidase F (PNGase F) were from New England Biolabs (Ipswich, MA, USA); ethanolamine and Nonidet P-40 were from Nacalai Tesque (Kyoto, Japan); horseradish peroxidase-linked anti-mouse and anti-rabbit IgG antibodies were from GE Healthcare (Piscataway, NJ, USA); anti-human NAAA rabbit polyclonal antibody recognizing the middle region of NAAA (anti-ASAHL antibody-middle region; catalog number ARP44939_P050) was from Aviva Systems Biology (San Diego, CA, USA); anti-FLAG M2 monoclonal antibody was from Sigma-Aldrich (St. Louis, MO, USA). Anti-rat NAAA rabbit polyclonal antiserum was prepared as described previously [22]. [^{14}C]Palmitoylethanolamide was chemically prepared from [^{14}C]palmitic acid and ethanolamine [27]. CMK cells were kindly provided by Dr. Eiji Shimizu of the Third Department of Internal Medicine, Tottori University School of Medicine, with the consent of Dr. Takeyuki Sato of Chiba University. An androgen-insensitive LNCaP subline (AILNCaP) [28] and VCaP cells were kindly provided by Drs. Yu Miyazaki and Takahiro Inoue of Kyoto University.

2.2. Cell culture

LNCaP, DU145, PC3, CMK, and THP-1 cells were cultured in RPMI 1640 medium containing phenol red. VCaP cells were cultured with DMEM/Ham's F12 containing phenol red. HeLa, MCF-7, and HEK293 cells were cultured with DMEM containing phenol red. To all these media were added 10% heat-inactivated FCS, penicillin (100 U/ml), and streptomycin (100 μg/ml). AILNCaP cells were maintained in phenol red-free RPMI 1640 medium with 10% charcoal stripped FCS, penicillin (100 U/ml), and streptomycin (100 μg/ml). PrEC cells were cultured in Prostate Epithelial Cell Basal Medium according to the manufacturer's instructions. All cells were cultured at 37 °C in a humidified 5% CO_2/95% air incubator.

2.3. Conventional reverse transcription-PCR

Total RNA was isolated from the cells by RNeasy mini kit. cDNA was then synthesized by the use of Moloney murine leukemia virus reverse transcriptase and random hexamer. To detect splice variants of human NAAA, PCR amplification was performed with Ex Taq DNA polymerase and primers shown in Tables 1 and 2. The condition was as follows: denaturation at 94 °C for 30 s, annealing at 67 °C for 30 s, and extension at 72 °C for 48 s (30 cycles). PCR products were electrophoresed on a 2.5%

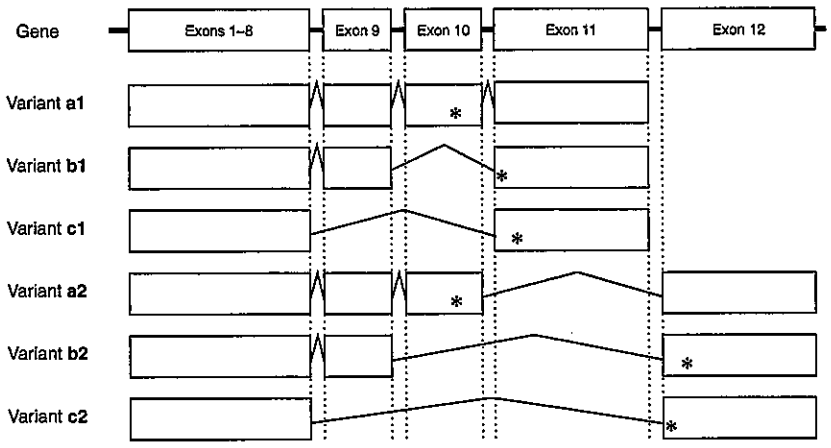

Fig. 1. Schematic representation of NAAA splice variants. * indicates stop codon.

Table 1
The sequences of PCR primers for NAAA.

Name	Exon(s)	Sequence
Forward primers		
f1	6	5′-GCTGATGTTTATTACATTGTTGGTGGCACGTC-3′
f5	8	5′-CAAACCTCAGCCTGGAGGC-3′
f7	8	5′-CAAGCAAACCTCAGCCTGGAGGC-3′
f11	1	5′-GCGG**AAGCTT**GAGCCCGAGCCATGCGGAC-3′
Reverse primers		
r2	8	5′-GAAAAGTGCCTCCAGGCTGAGGTTTG-3′
r3	11	5′-TCACTTTGTCTTATTTTTTAAGGTGCAGCTCTTCAAG-3′
r4	12	5′-CTGACAATCACCTGATGGGTTCTTCTTGCTC-3′
r6	10, 11	5′-CAGCTCTTCAAGAATTTCATTTTTTAAAAAATCATCTTTC-3′
r8	10, 12	5′-CAATCACCTGATGGGTTCTTCTTGCTCACTTT-3′
r9	9, 12	5′-CAATCACCTGATGGGTTCTTCTTGCTCATTGT-3′
r10	8, 12	5′-CCTGATGGGTTCTTCTTGCTCACTGG-3′
r12	9, 12	5′-GAGGGCCCTC**GAG**TCACCTGATGGGTTCTTCTTGCTCATTGTTA TAAACTGGAACCACCGACA-3′
r13	8, 12	5′-GAGGGCCCTC**GAG**TCACTGGAAAAGTGCCTCCAGGCTGAGG-3′

Underlined letters indicate nucleotides located in the forward exon, while the remaining nucleotides of the same primer are located in the backward exon. Bold letters indicate restriction sites: *Hin*dIII in f11, a combination of *Apa*I and *Xho*I in r12 and r13.

agarose gel and stained with ethidium bromide. For the sequencing of the splice variants, a1, a2, b2, and c2, PCR was similarly performed with PrimeSTAR DNA polymerase. After the agarose gel electrophoresis, the DNA bands corresponding to each splice variant were excised, and the DNAs were extracted from the gel and inserted into pCR4 Blunt-TOPO vector. The inserted DNAs were sequenced in both directions by an Applied Biosystems 3130 Genetic Analyzer (Thermo Fisher Scientific).

2.4. Real-time quantitative PCR (qPCR)

Total RNA was isolated from the cells by RNeasy mini kit. cDNAs were then synthesized with PrimeScript RT reagent kit. qPCR was carried out on an Applied Biosystems ViiA7 Real-Time PCR System (Thermo Fisher Scientific) by using SYBR Premix Ex Taq II. The primers used for NAAA splice variants are shown in Tables 1 and 2. PCR primers used for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were 5′-CGCTGAGTACGTCGTGGAGTCCACT-3′ (forward) and 5′-AGCAGA GGGGGCAGAGATGATGACC-3′ (reverse). The PCR conditions were as follows: for collective measurement of all NAAA splice variants and individual quantification of variants a2, b2, and c2, and GAPDH, denaturation at 95 °C for 5 s and annealing/extension at 63 °C for 30 s (40 cycles); for variant a1, denaturation at 95 °C for 5 s and annealing/extension at 60 °C for 30 s (40 cycles). Plasmid DNAs containing each splice variant prepared in Section 2.3 were linearized and used to draw standard curves by plotting the threshold cycles versus $\log_{10}$ of starting DNA quantities (copy numbers). These standard curves were used in order to compensate for the differences among amplification efficiencies (E) of each variant. Similarly, the standard curve for collective measurement of all splice variants was drawn with linearized variant a2-containing plasmid, and that for GAPDH was prepared with step-diluted LNCaP cDNAs. E values were estimated by the slopes of the standard curves according to the formula, $E\,(\%) = (10^{(-1\,/\,\text{slope})} - 1) \times 100$. E = 100% reflects a theoretically ideal amplification in which DNA amounts are doubled in each PCR cycle. The E values for variants a1, a2, b2, and c2 were 73.8 ± 4.5, 89.2 ± 0.8, 92.0 ± 7.1, and 91.1 ± 16.9 (%) (means ± S.D., $n = 2$), respectively. Those for collective measurement of all NAAA variants and for GAPDH were 97.3 ± 4.1 and 98.3 ± 9.4 (%) (means ± S.D., $n = 2$), respectively. The expression levels were normalized to GAPDH (a control).

Table 2
Combinations of forward and reverse primers used for amplification of NAAA splice variants.

Variant(s)	Forward primer	Reverse primer	Purpose
All variants	f1	r2	qPCR
a1, b1, and c1	f1	r3	Conventional PCR
a2, b2, and c2	f1	r4	Conventional PCR
a1	f5	r6	qPCR
a2	f7	r8	qPCR
b2	f1	r9	qPCR
c2	f1	r10	qPCR
b2	f11	r12	Plasmid construction
c2	f11	r13	Plasmid construction

2.5. Preparation of recombinant NAAA proteins

The cDNA encoding N-terminally FLAG-tagged isoform A of human NAAA, corresponding to splice variant a1, was prepared and inserted into the mammalian expression vector pcDNA3.1(+) at *Hin*dIII and *Apa*I sites as described previously [29]. This plasmid DNA was used as a template of PCR amplification to prepare cDNAs for isoforms B and C of NAAA with an N-terminal FLAG tag by using specific primers shown in Tables 1 and 2. The PCR products were inserted into pCR4 Blunt-TOPO vector, and further subcloned into pcDNA3.1(+) at *Hin*dIII and *Apa*I sites. For preparing cDNAs for point mutants of the FLAG-tagged isoform A, mutations were introduced into pcDNA3.1(+) harboring splice variant a1 of NAAA by PCR with the aid of a QuikChange site-directed mutagenesis kit. The primers used were listed in Table 3. The nucleotide sequences of all the constructs were determined in both directions to confirm the appropriate introductions of the inserts and mutations.

HEK293 cells were cultured at 37 °C to 70% confluency in a poly-L-lysine-coated 150-mm dish containing DMEM with 10% FCS and 0.1 mM nonessential amino acids in a humidified 5% CO_2/95% air incubator. The cells were transfected with 36 μg of pcDNA3.1(+) harboring cDNA for NAAA isoform A (wild-type or point mutants), B, or C by using 90 μl of Lipofectamine 2000 according to the manufacturer's instructions. Control cells were also prepared by transfection with the insert-free vector. After 48 h, the cells were harvested with the aid of trypsin, washed twice, suspended in 2.5 ml of phosphate-buffered saline (PBS), and sonicated three times each for 3 s. This sample was used as cell homogenate. The cell homogenate was centrifuged at 12,000 ×*g* for 30 min at 4 °C. The obtained pellet was resuspended in PBS and was subjected to two cycles of freezing and thawing, followed by further centrifugation at 105,000 ×*g* for 55 min at 4 °C. The resultant supernatant was used as solubilized proteins. Protein concentration was determined by the method of Bradford [30] with bovine serum albumin (BSA) as a standard.

Table 3
The sequences of PCR primers utilized to prepare point mutants of NAAA.

Name	PCR primers
L325A	F: 5′-GCACTTTTCCAGATTGCGTCGGT GGTTCCAG-3′ R: 5′-CTGGAACCACCGACGCAATCTG GAAAAGTGC-3′
L325T	F: 5′-GGCACTTTTCCAGATTACGTCGGT GGTTCCAG-3′ R: 5′-CTGGAACCACCGACGTAATCTG GAAAAGTGCC-3′
T335A	F: 5′-CCAGTTTATAACAACTTCGCAATTT ATACTACGGTAATGAGC-3′ R: 5′-GCTCATTACCGTAGTATAAAT TGCGAAGTTGTTATAAACTGG-3′
T335V	F: 5′-GTTCCAGTTTATAACAACT TCGTAATTTATACTACGGTAATGAGC-3′ R: 5′-GCTCATTACCGTAGTATAA ATTACGAAGTTGTTATAAACTGGAAC-3′

F and R refer to the forward and reverse PCR primers, respectively. Modified bases are in bold type.

2.6. Western blotting

To remove asparagine-linked oligosaccharide chains from NAAA protein, the samples (15–20 μg) were denatured in 0.5% SDS and 40 mM DTT at 100 °C for 10 min and treated with 500 units of PNGase F at 37 °C for 2 h in the presence of 1% Nonidet P-40. The samples were then separated by SDS-PAGE on a 14% gel under denaturing conditions, and proteins were electrotransferred to an Immobilon-P membrane. The following procedures were performed at room temperature. The membrane was blocked with 5% non-fat dried milk in PBS containing 0.1% Tween 20 for 1 h, and then incubated with anti-FLAG antibody (1:4000 dilution), anti-NAAA antibody (1:500 dilution), or anti-NAAA antiserum (1:2000 dilution) in the blocking buffer for 1 h, followed by the incubation with horseradish peroxidase-linked anti-mouse IgG (1:4000 dilution for FLAG) or anti-rabbit IgG (1:1000 and 1:4000 dilutions for anti-NAAA antibody and antiserum, respectively, as the first antibody) antibody in the blocking buffer for 1 h. Finally, after incubation with Pierce Western Blotting Substrate Plus, the membrane was exposed to an X-ray film.

2.7. Enzyme assay

Recombinant NAAA was allowed to react with 200 μM [-^{14}C]palmitoylethanolamide (1000 cpm/nmol, dissolved in 10 μl of dimethylsulfoxide) at 37 °C for 30 min in 100 μl of 100 mM citrate-Na_2HPO_4 (pH 4.5) containing 3 mM DTT, 0.1% Nonidet P-40, 0.05% BSA, and 150 mM NaCl. The reaction was terminated by the addition of 0.32 ml of a mixture of diethyl ether/methanol/1 M citric acid (30:4:1, by vol.). After centrifugation, 100 μl of the organic phase was spotted on a silica gel thin-layer plate (10-cm height) and developed at 4 °C for 25 min with a mixture of chloroform/methanol/28% ammonium hydroxide (80:20:2, by vol.). The radioactivities of the substrate and product on the plate were quantified by a Fujifilm FLA-7000 image analyzer (FUJIFILM Life Science, Tokyo, Japan). All the enzyme assays were performed in triplicate.

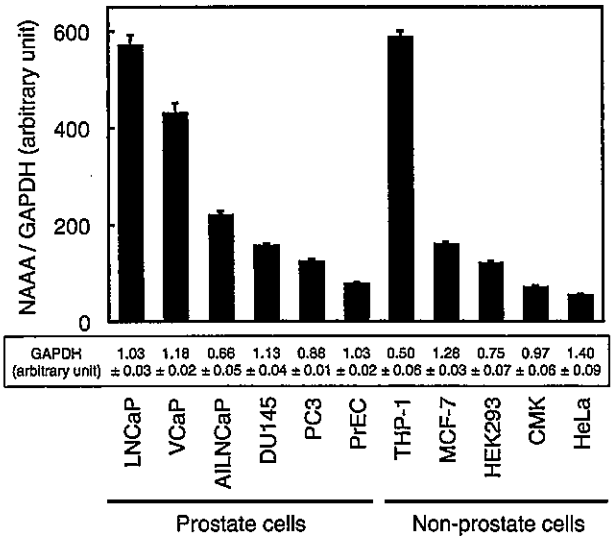

Fig. 2. Expression levels of NAAA mRNA in various human cells. The expression levels of NAAA mRNA in the indicated cells were measured by qPCR without distinguishing its splice variants as described in Materials and methods. The results were normalized to the levels of GAPDH mRNA. The GAPDH levels are also shown as boxed values. Values are expressed as means ± S.D. ($n = 3$).

3. Results

3.1. Collective measurement of all splice variants of NAAA mRNA

We first carried out qPCR to collectively quantify all splice variants of NAAA mRNA in various human cells, including five prostate cancer cells (LNCaP, VCaP, AILNCaP, DU145, and PC3), prostate epithelial cells (PrEC), and other cells: THP-1 (monocytic leukemia cells), MCF-7 (breast cancer cells), HEK293 (embryonic kidney cells), CMK (megakaryoblastic cells), and HeLa (cervical cancer cells) (Fig. 2). Since exons 1–8 of NAAA mRNA are common to all the splice variants, the combination of the forward primer f1 designed in exon 6 and the reverse primer r2 in exon 8 was expected to produce a single PCR product from all the splice variants (Tables 1 and 2). The results indicated that all the cell types examined express NAAA but its expression levels vary by cell type. When normalized to GAPDH levels, NAAA mRNA was most abundantly expressed in LNCaP, followed by VCaP cells, and their levels were higher than that in PrEC by 7.2- and 5.4-folds, respectively. Other prostate cancer cell lines, AILNCaP, DU145, and PC3, showed lower levels, which were less than 2.8-fold of that in PrEC. Among the non-prostate cells examined, THP-1 highly expressed NAAA. The differences among the GAPDH levels were within 2.8-fold (boxed values in Fig. 2). To examine the batch-to-batch differences, LNCaP and HEK293 cells amplified from three different cryostocks were analyzed as representatives of the prostate and non-prostate cell lines, respectively. The values were 614 ± 191 and 133 ± 14 (means ± S.D., $n = 3$), respectively.

3.2. Identification of NAAA splice variants in LNCaP cells

Considering the high expression level of NAAA mRNA in LNCaP cells, we next performed conventional reverse transcription-PCR to amplify splice variants of NAAA from LNCaP cells and determined their nucleotide sequences. For this PCR, we used the forward primer f1 (in exon 6) shared by all splice variants and one of the reverse primers r3 (in exon 11) and r4 (in exon 12) to separately detect the exon 11-containing variants (a1, b1, and c1 in Fig. 1) and exon 12-containing variants (a2, b2, and c2) (Table 2). As shown in Fig. 3, four major PCR products (one product on lane 1 and three products on lane 2) were observed and identified as the splice variants a1, a2, b2, and c2 by nucleotide sequencing. Some minor bands on lane 1, which might include b1 and c1, remained unidentified. The levels of these minor PCR products were also low in other prostate cancer cells (VCaP, AILNCaP, DU145, and PC3) and prostate epithelial cells (PrEC) (data not shown).

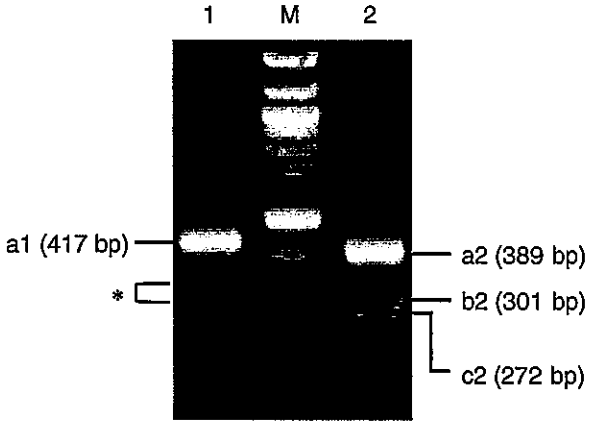

Fig. 3. Expression of NAAA splice variants in LNCaP cells. Expression of NAAA splice variants in LNCaP cells was examined by conventional PCR as described in Materials and methods. The reverse primers used were r3 (lane 1) and r4 (lane 2). 100 bp DNA Ladder was applied (lane M). * indicates unidentified bands.

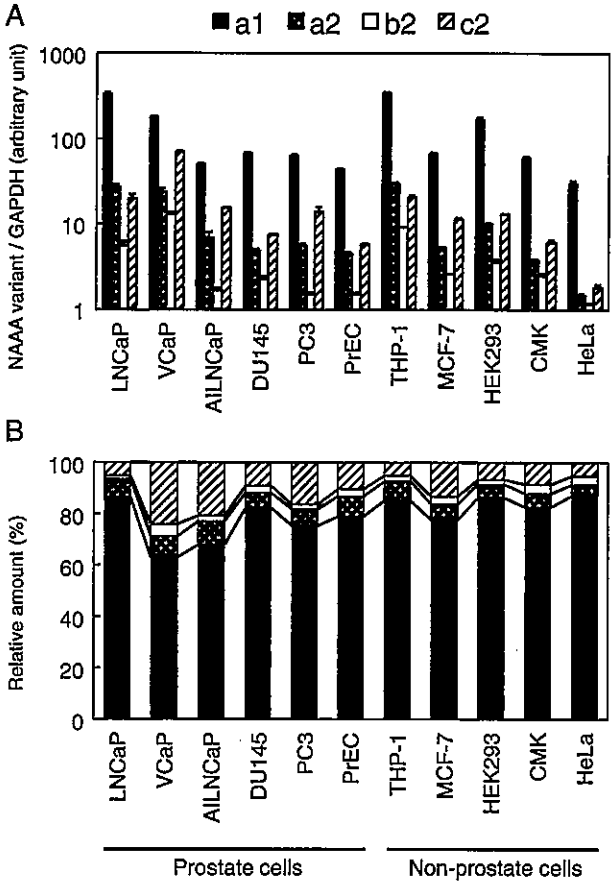

Fig. 4. Expression levels of major splice variants of NAAA mRNA in various human cells. (A) The expression levels of the splice variants a1, a2, b2, and c2 in the indicated cells were quantified by qPCR as described in Materials and methods. The results were normalized to the levels of GAPDH mRNA (means ± S.D., n = 3). (B) The same results are shown as relative abundance of the four variants in each cell type.

3.3. qPCR analysis of NAAA splice variants

We developed a qPCR method to individually quantify splice variants a1, a2, b2, and c2. For this purpose, we designed four reverse primers r6, r8, r9, and r10, which can distinguish one variant from the other three variants by simultaneously recognizing two adjacent exons (Tables 1 and 2). As analyzed by this method, all the cell types were found to express all of a1, a2, b2, and c2. After the normalization to GAPDH, variant a1 always showed the highest expression levels, while the levels of variant b2 were constantly the lowest (Fig. 4A). However, proportions of each variant in total amount of the four variants were different by cell type (Fig. 4B). When compared among prostate cancer cells, the proportion of variant c2 was high in VCaP, AILNCaP, and PC3 (16–24%) and low in LNCaP and DU145 (5–9%). Variant c2 accounted for 10% in PrEC. We also analyzed commercially available human normal prostate tissue. The ratio of a1:a2:b2:c2 was 64:5:3:28. This ratio was similar to those of the prostate cancer cells, although the proportion of variant c2 (28%) was the highest in the normal prostate tissue. We next examined the batch-to-batch differences by using LNCaP and HEK293 cells derived from three different cryostocks. The proportions of variants a1, a2, b2, and c2 in LNCaP cells were 89.3 ± 3.0, 4.7 ± 1.5, 1.1 ± 0.3, and 4.8 ± 1.2 (%) (means ± S.D., $n = 3$), respectively, and those in HEK293 cells were 86.0 ± 1.7, 5.3 ± 0.4, 1.6 ± 0.2, and 7.1 ± 1.2 (%) (means ± S.D., $n = 3$). These results showed that the batch-to-batch differences were small.

3.4. Characterization of recombinant proteins translated from splice variants

Since the stop codon of variants a1 and a2 exists in exon 10 (Fig. 1), both variants should be translated to the same NAAA protein composed of amino acids 1–359 (referred to as isoform A in this article). On the other hand, variants b2 and c2 should be translated to the C-terminally truncated forms composed of 1–340 and 1–323, respectively (isoforms B and C) (Fig. 5). Variant b2 lacks exons 10 and 11 and possesses exon 12, which contains the sequence corresponding to amino acids 334–340 and the stop codon, while in the case of variants a1 and a2 amino acids 334–359 are encoded in exon 10 (Fig. 1). Thus, the sequences of amino acids 334–340 are different between isoforms A and B. However, the primary structure of isoform C is identical to the corresponding sequences of isoforms A and B due to the lack of exons 9–11 and the start of exon 12 with a stop codon in variant c2. In the course of posttranslational processing, human NAAA (isoform A) is first formed as a catalytically inactive precursor consisting of amino acids 29–359 devoid of the signal peptide 1–28 and then autocatalytically cleaved between Phe-125 and Cys-126 at acidic pH to generate a mature form consisting of amino acids 126–359 [29].

To characterize these protein isoforms, we introduced cDNAs of a1, b2, and c2 into HEK293 cells by lipofection method and overexpressed recombinant NAAA proteins with a FLAG tag at N terminus. After the sugar chains of NAAA were removed with *N*-glycanase, the cell homogenates containing each isoform were analyzed by Western blotting. As immunostained with anti-FLAG antibody, the precursors of isoforms A, B, and C were detected around the positions of the expected molecular masses (38.2, 36.1, and 34.1 kDa, respectively) (Fig. 6A). The mature form of NAAA can be solubilized from the 12,000 ×*g* pellet by freezing and thawing [13,22]. Since the mature form lost the N-terminal FLAG tag, we used commercially available anti-NAAA antibody raised against amino acids 216–265 in order to detect the mature form of isoforms A, B, and C in the solubilized protein fraction. The result showed that the mature form of isoform A (calculated to be 26.3 kDa) was present, but those of isoforms B (24.2 kDa) and C (22.2 kDa) were undetectable (Fig. 6B). Moreover, different from the catalytically active isoform A, neither isoform B nor C exhibited [^{14}C]palmitoylethanolamide-

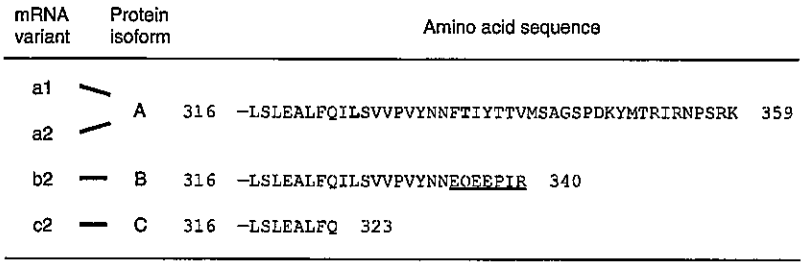

Fig. 5. Amino acid sequences of NAAA isoforms. Amino acids 1–315 common in all isoforms are pretermitted. Underlined letters indicate amino acids of isoform B, which are different from those of isoform A. The amino acids of isoform A shown by bold letters were subjected to point mutation in the experiments of Fig. 7.

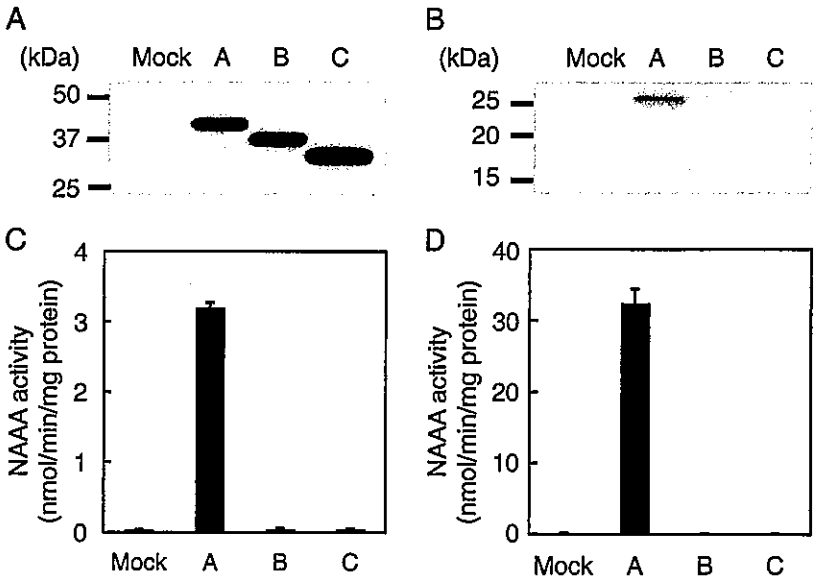

Fig. 6. Western blotting and enzyme assay of recombinant NAAA isoforms. After digestion with PNGase F, the homogenates (5 μg protein) of HEK293 cells expressing the indicated recombinant NAAA isoforms were subjected to Western blotting with anti-FLAG antibody (A). Their solubilized protein fractions (12 μg protein) were analyzed with anti-NAAA antibody (B). The homogenates (25 μg protein) (C) and the solubilized protein fractions (2 μg protein) (D) were also subjected to enzyme assay as described in Materials and methods. The activities are shown as mean values ± S.D. ($n = 3$). The samples derived from the cells transfected with the insert-free vector were used as negative controls (Mock).

hydrolyzing activity in both homogenates (Fig. 6C) and solubilized protein fractions (Fig. 6D). These results suggested that isoforms B and C are catalytically inactive despite the generation of their precursor forms.

3.5. Characterization of the point mutants of recombinant NAAA

Since the aforementioned results suggested the presence of catalytically important amino acid residues in the C-terminal region of isoform A (amino acids 324–359), we next tried to identify these residues. We focused on Leu-325 and Thr-335 because they are highly conserved within the cysteine-type N-terminal nucleophile (Ntn) hydrolase family including NAAA [31,32]. Moreover, their corresponding amino acids were located near the catalytic center of penicillin V acylase, a member of the family [32]. We then prepared four point mutants of FLAG-tagged isoform A (L325A, L325T, T335A, and T335V), and overexpressed them in HEK293 cells. When the cell homogenates containing each mutant were analyzed by Western blotting with anti-FLAG antibody, the expressions of all the precursors were confirmed by detecting immunopositive bands around the positions of the expected molecular masses (38.2 kDa) (Fig. 7A). We next used anti-NAAA antiserum which could recognize both of the precursor and mature forms of isoform A [29]. Whereas the mature form of wild-type isoform A (calculated to be 26.3 kDa) was clearly observed, those of the point mutants were faintly stained (L325A and L325T) or undetectable (T335A and T335V) (Fig. 7B). The precursor forms (38.2 kDa) of all mutants were detected with this antiserum, similar to the observation with anti-FLAG antibody. In accordance with these results of Western blotting, the [^{14}C]palmitoylethanolamide-hydrolyzing activities of the mutants were much lower than that of wild-type isoform A (L325A and L325T) or almost undetectable (T335A and T335V) (Fig. 7C). These results suggested that Leu-325 and Thr-335 are catalytically important and that the latter one is indispensable for the enzyme activity of NAAA.

4. Discussion

A series of our previous studies revealed that NAAA is a lysosomal glycoprotein which catalyzes hydrolysis of various bioactive *N*-acylethanolamines such as palmitoylethanolamide to the corresponding fatty acids and ethanolamine [15,22,29]. The characterization of human NAAA has been performed with recombinant protein biosynthesized from the cDNA encoding amino acids 1–359 (isoform A in the present study). However, prior to molecular identification of NAAA [15], Goodchild et al. reported that multiple transcripts are produced from *NAAA* gene by alternative splicing [26]. They screened a cDNA library from a normal human placenta in attempt to clone cDNA containing the long terminal repeats (LTRs) and found a clone cPj-LTR, which contains LTR at the 3′-terminus. cPj-LTR corresponded to variant c2 of NAAA, and LTR existed in exon 12. Concurrently, the sequences corresponding to variants a1 and b1 were also found as 3′-end splice variants in placenta. As examined by Northern blotting, cPj-LTR was detected in placenta, but not in the various cell lines examined. Later, Hong et al. reported cDNA cloning of NAAA [25]. Since the function of the protein was unknown at that time, they named this protein "acid ceramidase-like protein". Its nucleotide sequence was identical to variant a1, and they did not refer to other splice variants. Thus, tissue distribution of each NAAA splice variant remained unclear, and quantitative methods for this purpose have not been developed.

We earlier found NAAA activity in CMK cells [13] and later used this cell line to clone cDNA of human NAAA [15]. By using conventional PCR method, we also reported wide distribution of NAAA mRNA in human tissues with the highest expression level in prostate [23]. As for established human cell lines, we showed the expression of NAAA mRNA in monocyte-like cells THP-1 and U937 [21] and prostate cancer cells LNCaP, DU145, and PC3 [23]. Since we used a forward primer designed in exon 3 and a reverse primer in exon 6 for PCR in those studies, all NAAA variants were presumably amplified as a single product. The present study confirmed the expression of NAAA mRNA in CMK, THP-1, LNCaP, DU145, and PC3. We also found that all of other human cells tested express NAAA mRNA.

It is known that the majority of prostate cancers are sensitive to androgen and the androgen ablation therapy at their initial stages results in temporary shrinkage or interruption in the growth of tumors [33]. However, in some cases the cancer acquires the ability to grow in the absence of hormones. Prostate cancer cell lines so far established can be classified according to the sensitivity to androgen: LNCaP and VCaP are androgen-sensitive cells, while AILNCaP, DU145, and PC3 are

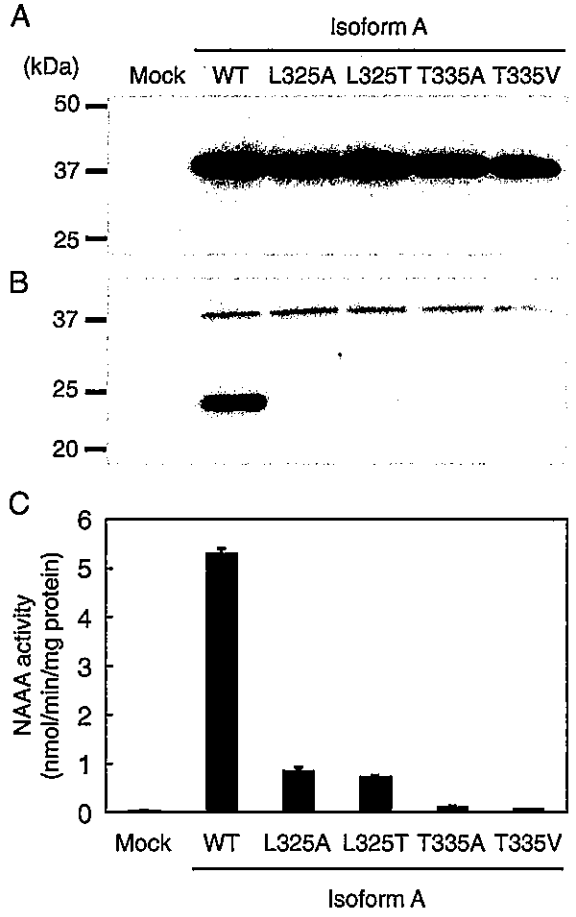

Fig. 7. Western blotting and enzyme assay of point mutants of NAAA isoform A. After digestion with PNGase F, the homogenates (5 μg protein) of HEK293 cells expressing the indicated point mutants of NAAA isoform A were subjected to Western blotting with anti-FLAG antibody (A) and anti-NAAA antiserum (B). The homogenates (25 μg protein) were also subjected to enzyme assay (C). The activities are shown as mean values ± S.D. ($n = 3$). The samples derived from the control cells (Mock) and wild-type isoform A-expressing cells (WT) were used as negative and positive controls, respectively.

androgen-insensitive cells [33,34]. AILNCaP, a subline of LNCaP, was developed from LNCaP by the long-term culture in the absence of androgen to obtain androgen-independency. In the present study we showed that the total levels of NAAA mRNAs were higher in the two androgen-sensitive cells and lower in the three androgen-insensitive cells. This result suggests the potential usefulness of NAAA as a biomarker of androgen sensitivity.

Our main purpose in the present study was not to identify all of NAAA variants, but to investigate whether the proportion of multiple variants differs across the cells of different characteristics. We, therefore, focused on the four variants (a1, a2, b2, and c2), which were highly expressed in LNCaP and other prostate cancer cells, and developed qPCR-based quantitative methods for each variant. We did not measure b1, b2, or variants lacking both exons 11 and 12, which were reported previously in placenta [26] or found on NCBI database. Our results showed that these four variants are present in all the cell types examined and that variant a1 is constantly the most abundant (63–87% of total amount of the four variants). Thus, although both a1 and a2 encode the functionally active full-length NAAA protein (isoform A), it was suggested that a1 principally contributes to the translation to isoform A. As was expected, the proportion of each variant differed by cell type. For example, the proportion of c2 (5–24%) was different by each cell type. However, an obvious difference in the proportion of each variant was not seen between prostate cells and non-prostate cells or between androgen-sensitive and -insensitive prostate cancer cells.

Variants b2 and c2 were constantly present as minor components and could be translated to the truncated forms of NAAA (isoforms B and C) as overexpressed in HEK293 cells. Although Western blot analysis confirmed the expression of the precursors of all isoforms in the cell homogenates, we could not find the mature forms of isoforms B and C in the solubilized protein fractions in which lysosomal matrix proteins were expected to exist. In addition, [^{14}C]palmitoylethanolamide-hydrolyzing activity was not seen with these isoforms. These results suggested that the maturation by autocatalyzed proteolytic cleavage does not occur in isoforms B or C. Previously, it was reported that Cys-126, Arg-142, Asp-145, and Asn-287 are highly conserved within the cysteine-type Ntn hydrolase family, including NAAA and acid ceramidase [31,32] and indispensable for the maturation by autocatalyzed cleavage [35–37]. These four amino acid residues were conserved in isoforms B and C. In the present study, we showed that Leu-325 and Thr-335 are also important for the self-catalyzed maturation. Since isoform C lacked both the amino acids and since isoform B lacked Thr-335, unsuccessful maturation of these isoforms was considered to be due to the loss of these amino acids. It was also possible that the produced mature forms were unstable and rapidly degraded in the cell. It was reported that the *N*-glycosylations at Asn-37, Asn-107, Asn-309, and Asn-333 play an important role in stabilizing NAAA protein [29]. Isoform C lacked Asn-333. Isoform B kept this asparagine, but lost a consensus sequence for *N*-glycosylation site (Asn-Xaa-Ser/Thr) because Thr-335 was changed to Gln. The deficiency of the sugar chain at Asn-333 might destabilize these isoforms.

NAAA is responsible for the degradation of bioactive *N*-acylethanolamines and is abundantly expressed in prostate cancer cells as well as normal prostate tissue [23]. Specific NAAA inhibitors were actually shown to increase endogenous *N*-acylethanolamine levels [18–20]. Palmitoylethanolamide was reported to exert an anti-proliferative effect on human colon carcinoma cells [38]. Endogenous levels of endocannabinoids (anandamide and 2-arachidonoylglycerol) were elevated in several cancer cells including prostate, compared with their normal counterparts [39,40]. Cannabinoid receptor agonist sustained the activation of ERK1/2 in LNCaP cells. Consequently, G1 cell cycle arrest occurred and apoptosis of the cancer cells was induced [41]. Furthermore, cannabinoid receptor CB1 immunoreactivity was correlated with the malignancy of prostate cancer [42]. These findings show a possibility that NAAA regulates the growth of prostate cancer by lowering the endogenous levels of *N*-acylethanolamines.

Recently, the C-terminally truncated splice variant of androgen receptor, called AR-v7, has received attention as a biomarker for advanced prostate cancer. Since this variant lacks the ligand-binding domain, the activation spontaneously occurs irrespective of androgen binding, resulting in the promotion of prostate cancer proliferation [43]. Although we found that the proportions of catalytically inactive NAAA variants (b2 and c2) are higher in some cells, it remained unclear whether this change affects endogenous *N*-acylethanolamine levels. Further study may give a clue as for pathophysiological significance of NAAA variants. Interestingly, according to NCBI database, there was no evidence that mouse NAAA mRNA possesses multiple splice variants. Acid ceramidase has a gene structure analogous to NAAA [25]. However, human acid ceramidase possessed four 5′-end splice variants (accession numbers NM_004315.4, NM_001127505.1, NM_177924.3, and XM_005273504.2), but not 3′-end splice variants.

In conclusion, we demonstrated the presence of multiple splice variants of NAAA mRNA in various human cells. We also showed that all the cells tested express not only the variants a1 and a2, which should be translated to catalytically active NAAA protein, but also the variants b2 and c2, corresponding to catalytically inactive proteins, and that the proportion of the four splice variants is different among various cell types.

Transparency document

The Transparency document associated with this article can be found, in the online version.

Acknowledgments

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