Molecular Characterization of N-Acylethanolamine-hydrolyzing Acid Amidase, a Novel Member of the Choloylglycine Hydrolase Family with Structural and Functional Similarity to Acid Ceramidase*

Kazuhito Tsuibo†, Yong-Xin Sun‡, Yasuo Okamoto‡, Nobukazu Araki§, Takeharu Tonai¶, and Natsu Ueda∥

From the †Departments of Biochemistry and Histology and Cell Biology, Kagawa University School of Medicine, 1750-1 Ikenobe, Miki, Kagawa 761-0793 and the ¶Department of Orthopedic Surgery and Clinical Research Institute, National Zentsuji Hospital, Zentsuji, Kagawa 765-0001, Japan

Bioactive N-acylethanolamines, including anandamide (an endocannabinoid) and N-palmitoylethanolamine (an anti-inflammatory and neuroprotective substance), are hydrolyzed to fatty acids and ethanolamine by fatty acid amidase hydrodrolase. Moreover, we found another amidohydrolase catalyzing the same reaction only at acidic pH, and we purified it from rat lung (Ueda, N., Yamanaka, K., and Yamamoto, S. (2001) J. Biol. Chem. 276, 35552–35557). Here we report complementary DNA cloning and functional expression of the enzyme termed “N-acylethanolamine-hydrolyzing acid amidase (NAAA)” from human, rat, and mouse. The deduced primary structures revealed that NAAA had no homology to fatty acid amidase hydrodrolase but belonged to the choloylglycine hydrolase family. Human NAAA was essentially identical to a gene product that had been noted to resemble acid ceramidase but lacked ceramide hydrolyzing activity. The recombinant human NAAA overexpressed in HEK293 cells hydrolyzed various N-acylethanolamines with N-palmitoylethanolamine as the most reactive substrate. Most interestingly, a very low ceramide hydrolyzing activity was also detected with NAAA, and N-lauroylethanolamine hydrolyzing activity was observed with acid ceramidase. By the use of tunicamycin and endoglycosidase, NAAA was found to be a glycoprotein. Furthermore, the enzyme was proteolytically processed to a smaller form at pH 4.5 but not at pH 7.4. Expression analysis of a green fluorescent protein-NAAA fusion protein showed a lysosome-like distribution in HEK293 cells. The organ distribution of the messenger RNA in rats revealed its wide distribution with the highest expression in lung. These results demonstrated that NAAA is a novel N-acylethanolamine-hydrolyzing enzyme that shows structural and functional similarity to acid ceramidase.

Ethanolamides of long-chain fatty acids, collectively referred to as N-acylethanolamines (NAEs), are ubiquitously present in a variety of organisms (1–4). Earlier, saturated and monounsaturated NAEs including N-palmitoylethanolamine, N-stearoylethanolamine, and N-oleoylethanolamine were found as major NAEs in mammalian tissues (5, 6). Later, polyunsaturated NAEs represented by N-arachidonoylethanolamine (anandamide) were identified as endogenous ligands of cannabinoid receptor CB1 (7, 8). Anandamide was reported to show a variety of cannabinimetic activities (9) and also to act as a ligand of the transient receptor potential vanilloid type 1 (10). Saturated and unsaturated NAEs are inactive as ligands of cannabinoid receptors but have been reported to reveal various biological activities (1, 11, 12). N-Palmitoylethanolamine has anti-inflammatory (13–15), anti-nociceptive (16, 17), immunosuppressive (18), neuroprotective (19), and antioxidant (20) effects. Recently, it was shown that the anti-inflammatory action of N-palmitoylethanolamine could be mediated by the activation of peroxisome proliferator-activated receptor-α (PPAR-α) (21). Furthermore, N-oleoylethanolamine was shown to be anorexic via PPAR-α (22, 23) and N-stearoylethanolamine to be pro-apoptotic (24) and anorexic (25). In addition, NAEs have been noted to increase markedly in degenerating tissues and cells (6, 26–28). We recently cloned cDNA of a novel phospholipase D that specifically releases NAEs from their corresponding N-acylphosphatidylethanolamines (29), further supporting the physiological significance of NAEs.

Anandamide and other NAEs are hydrolyzed to free fatty acids and ethanolamine, and this intracellular degradation is mostly attributed to the catalysis by fatty acid amidase hydrolase (FAAH) (30–33). This membrane-bound enzyme is widely distributed in mammalian organs and is characterized by an optimal pH value at 8.5–10 and high sensitivity to serine phospholipase A that specifically releases NAEs from their corresponding N-acylphosphatidylethanolamines (29), further supporting the physiological significance of NAEs.

1 The abbreviations used are: NAE, N-acylethanolamine; AC, acid ceramidase; BSA, bovine serum albumin; DMM, Dulbecco’s modified Eagle medium; DTT, dithiothreitol; FAAH, fatty acid amidase hydrolase; OCS, fetal calf serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescent protein; HEK293, human embryonic kidney 293; NAAA, N-acylethanolamine-hydrolyzing acid amidase; PBS, phosphate-buffered saline; PNGase F, peptide: N-glycosidase F; PPAR-α, peroxisome proliferator-activated receptor-α; RT, reverse transcription.

* This work was supported in part by grants-in-aid for scientific research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (to K. T.), Japan Society for the Promotion of Science (to N. A.), and Kagawa Medical University (to K. T.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the DDBJ/GenBank®/EBI Data Bank with accession number(s) AB161353, AB162193, and AB162194.

† To whom correspondence should be addressed: Dept. of Biochemistry, Kagawa University School of Medicine, 1750-1 Ikenobe, Miki, Kagawa 761-0793, Japan. Tel.: 81-87-891-2102; Fax: 81-87-891-2105; E-mail: nueda@med.kagawa-u.ac.jp.
A Second N-Acylethanolamine Hydrodase and Acid Ceramidase

11083

(34) and crystallography (35), revealed that FAAH belongs to the amidase signature family and possesses a catalytic triad of Ser-Ser-Lys. In addition, analysis of FAAH-deficient mice showed the central role of this enzyme in the degradation of anandamide and other NAEs in the brain (36–38).

In addition to FAAH, we found another NAE-hydrolyzing amidohydrolase, which was active only at acidic pH, first in human megakaryoblastic (CMK) cells (39) and later in various rat tissues including lung, spleen, and macrophages (40). This enzyme, termed as N-acylethanolamine-hydrolyzing acid amidase (NAAA) in the present article, was distinguishable from FAAH by its optimal pH around 5, the preference of N-palmitoyl ethanolamine to other NAEs, activation by Triton X-100 (a non-ionic detergent) and dithiothreitol (DTT), and lower sensitivity to phenylmethylsulfonyl fluoride and methyl arachidonyl fluorophosphonate (39, 40). We recently found that some ester and amide compounds such as N-cyclohexanecarbonylpentadecylamine are selective inhibitors of NAAA without inhibitory effects on FAAH (41, 42). These results strongly suggested that NAAA was an enzyme protein different from FAAH.

The molecular characterization of NAAA was indispensable to the elucidation of its physiological role. Although we purified NAAA from rat lung to apparent homogeneity (40), cDNA cloning of NAAA has not been performed to date. Here we report for the first time the identification of cDNA encoding NAAA. The functional expression of the cDNA confirmed NAAA to be a second NAE-hydrolyzing enzyme. Furthermore, the deduced amino acid sequence unveiled similarity of NAAA to acid ceramidase (AC), an amidohydrolase-hydrolyzing ceramide to sphingosine and fatty acid, which urged us to compare catalytic properties of NAAA with those of AC. We also investigated possible glycosylation and proteolytic modification of NAAA and its intracellular localization. Our results may contribute to the further understanding of the catabolism of NAEs and ceramides in the context of lipid metabolism.

EXPERIMENTAL PROCEDURES

Materials—[1-14C]Palmitic acid was purchased from PerkinElmer Life Sciences; [1-14C]myristic acid was from Moravek Biochemicals (Brea, CA); [1-14C]lauric, [1-14C]stearic, [1-14C]oleic, and [1-14C]arachidonic acids, phenyl-Sepharose CL-4B, HiTrap heparin HP, HiTrap butyl FF, Hybond-P, ECL reagents, and Ready-To-Go You-Prime First-strand Beads. For cDNA cloning of human NAAA, we prepared two cDNA fragments according to the cDNA sequence of acid ceramidase-like protein (46). One cDNA fragment was amplified by LA Taq polymerase with the sense primer 5'-AAGACTCCAGAGGCCACATTT-GAGGAATTCCAGT-3' and the antisense primer 5'-AGGAATTCCAGT-3 for mouse; the antisense primer 5'-GCGGAC-3' for rat or 5'-CTCTACTCCGGTTTTC-3' for mouse NAAA cDNA. For cDNA cloning of rat NAAA, we first amplified cDNA fragments according to the cDNA sequence of acylceramidase-like protein (46). One cDNA fragment was amplified by LA Taq polymerase with the sense primer 5'-GCGGAC-3' and the antisense primer 5'-ATCAGCTTGGACGTTG-3' for rat or 5'-GAGTACCTCAGGTACGTTTGGC-3' (mouse), and the antisense primer 5'-TTCTCTCTGAGTTATCTC-3' for the human cDNA. For cDNA cloning of rat and mouse NAAA, we first amplified cDNA fragments according to the cDNA sequence of acylceramidase-like protein (46). One cDNA fragment was amplified by LA Taq polymerase with the sense primer 5'-GCGGAC-3' and the antisense primer 5'-ATCAGCTTGGACGTTG-3' for rat or 5'-GAGTACCTCAGGTACGTTTGGC-3' (mouse), and the antisense primer 5'-TTCTCTCTGAGTTATCTC-3' for the human cDNA. For cDNA cloning of rat and mouse NAAA, we first amplified cDNA fragments according to the cDNA sequence of acylceramidase-like protein (46). One cDNA fragment was amplified by LA Taq polymerase with the sense primer 5'-GCGGAC-3' and the antisense primer 5'-ATCAGCTTGGACGTTG-3' for rat or 5'-GAGTACCTCAGGTACGTTTGGC-3' (mouse), and the antisense primer 5'-TTCTCTCTGAGTTATCTC-3' for the human cDNA. For cDNA cloning of rat and mouse NAAA, we first amplified cDNA fragments according to the cDNA sequence of acylceramidase-like protein (46). One cDNA fragment was amplified by LA Taq polymerase with the sense primer 5'-GCGGAC-3' and the antisense primer 5'-ATCAGCTTGGACGTTG-3' for rat or 5'-GAGTACCTCAGGTACGTTTGGC-3' (mouse), and the antisense primer 5'-TTCTCTCTGAGTTATCTC-3' for the human cDNA.
Identification and Cloning of NAAA cDNA—As described previously (40), we solubilized the NAAA enzyme from the 12,000 × g pellet of rat lung homogenates by freezing and thawing, and we purified it by acid treatment and four chromatographic steps using phenyl-Sepharose, HiTrap heparin, hydroxyapatite, and HiTrap butyl. Through this purification procedure, the specific enzyme activity was increased 780-fold from 5.1 nmol/min/mg protein to 3.9 μmol/min/mg protein with N-palmitoylthanolamine as substrate. In agreement with our previous results (40), the purified enzyme preparation gave a major protein band around 31 kDa as analyzed by SDS-PAGE. This 31-kDa protein was transferred to a hydrophobic polyvinylidene difluoride membrane (Hybond-P) and subjected to Western Blotting using an ECL kit.

RESULTS

Identification and Cloning of NAAA cDNA—As described previously (40), we solubilized the NAAA enzyme from the 12,000 × g pellet of rat lung homogenates by freezing and thawing, and we purified it by acid treatment and four chromatographic steps using phenyl-Sepharose, HiTrap heparin, hydroxyapatite, and HiTrap butyl. Through this purification procedure, the specific enzyme activity was increased 780-fold from 5.1 nmol/min/mg protein to 3.9 μmol/min/mg protein with N-palmitoylthanolamine as substrate. In agreement with our previous results (40), the purified enzyme preparation gave a major protein band around 31 kDa as analyzed by SDS-PAGE. This 31-kDa protein was transferred to a hydrophobic polyvinylidene difluoride membrane (Hybond-P) and subjected to Western Blotting using an ECL kit.
centa and characterized by Hong et al. (46). They named the protein as acid ceramidase-like protein because of the similarity of its primary structure to AC, an enzyme hydrolyzing ceramide to sphingosine and fatty acid at acidic pH. However, they reported that this protein had no detectable ceramide hydrolyzing activity, and its function remained unclear. Our result therefore suggested that NAAA was identical to acid ceramidase-like protein. We carried out RT-PCR with PCR primers based on the nucleotide sequences, and we cloned cDNAs of putative human, rat, and mouse NAAA (hNAAA, rNAAA, and mNAAA, respectively) from human megakaryoblastic CMK cells and lungs of rat and mouse in which we had found NAAA activity (39, 40).

We sequenced the coding region of hNAAA, which was found to be 99.8% identical at the nucleotide level to the acid ceramidase-like protein reported previously (46). The coding regions of rNAAA and mNAAA were 99.9% identical to the nucleotide sequences of XM_223237.2 and AK008776.1 (GenBankTM), respectively. The amino acid sequences deduced from the cDNAs are aligned in Fig. 1A. The amino acid sequences were composed of 359 (human) and 362 (rat and mouse) residues. The molecular masses were calculated to be 40,065 (human), 40,312 (rat), and 40,074 (mouse). Their amino acid identity was 76.5% (between human and rat), 76.7% (between human and mouse), and 90.1% (between rat and mouse). There were six potential N-glycosylation sites in the human sequences (denoted with asterisks, dots, and closed circles in Fig. 1A).

### Table 1: Amino acid sequences deduced from cDNAs for human, rat, and mouse NAAA

| Protein   | Amino Acid Sequence | Human | Rat | Mouse |
|-----------|---------------------|-------|-----|-------|
| hNAAA     | MRTDAREARPGLPSLLLLLLALAGSLSAASPPAPRNPVNSLDSVEPLLWLVHRYDDLVLRAAMQV | 71    | 71  | 71    |
| rNAAA     | MGTPIAARACHGHALALALLALLLSLPDLWATAGGPTPPFLPSLDAAPILEWLPMQHYDDPDDFVRAAQAQV | 76    | 76  | 76    |
| mNAAA     | MGTLLATRAACHGHALALALLALLLSLPGLPSLAVVGFPIPPFLPSLDAAPQWRPLMLPHYDDPFTTAVQAQV | 76    | 76  | 76    |
| hNAAA     | VPKWVHILGKVVELERELFPLQPTGPEIRGMCDMFPLSALACDIALVNLAYESVSFCTSIVAQDSRGHIGNLTDY | 147   | 147 | 147   |
| rNAAA     | VNPWILEMEVIEIVKQVVLQVFQPTAFASTVEILNLASGLCVLNLAYASAFCTSIVAQDSQGRISIHMNLTDY | 152   | 152 | 152   |
| mNAAA     | VPKWVHILGKVVELERELFPLQPTGPEIRGMCDMFPLSALACDIALVNLAYESVSFCTSIVAQDSRGHIGNLTDY | 152   | 152 | 152   |
| hNAAA     | PGNLRLKTLAVVQVAQNLHELALFSFVQVYNNFTITTYYTTMGA3PSDPKYMTRINPSRK | 359   | 359 | 359   |
| rNAAA     | PFSLPAKALNATQQAHLLELFQVFLSVFVYNNFTITTYYTTMGA3APKYMTRINPSRK | 362   | 362 | 362   |
| mNAAA     | PFSLPAKALNATQQAHLLELFQVFLSVFVYNNFTITTYYTTMGA3APKYMTRINPSRK | 362   | 362 | 362   |

### Fig. 1. Amino acid sequences deduced from cDNAs for human, rat, and mouse NAAA

A. Alignment of the amino acid sequences deduced from hNAAA, rNAAA, and mNAAA. Asterisks, dots, and closed circles indicate identity shared by three polypeptides, identity shared by two polypeptides, and the potential N-glycosylation sites of the human sequence, respectively. B. Comparison between amino acid sequences deduced from hNAAA and human AC. Asterisks and dots indicate identity and similarity, respectively. Dashes denote deletion of amino acid residues when compared with the other sequence. An arrowhead indicates the cleavage site to generate α- and β-subunits of human AC. Underlines in A and B denote the sequence corresponding with the N-terminal sequence of the purified rat NAAA protein, which we determined.
The amino acid sequences of hNAAA, rNAAA, and mNAAA showed no homology with human, rat, and mouse FAAH (34, 50) but revealed similarity to human AC (U70063) (47), rat AC (AF214647), and mouse AC (AF157500) (51), respectively. Those of hNAAA and human AC are aligned in Fig. 1B, which showed 33% identity and 70% similarity over their entire length. Likewise, the amino acid sequences of rNAAA and rat AC showed 33% identity and 70% similarity, and those of mNAAA and mouse AC showed 34% identity and 70% similarity. The data base search also revealed that NAAA as well as AC belongs to the chooloylglycine hydrolase family, which includes several hydrolases cleaving carbon-nitrogen bonds, other than peptide bonds, in linear amides (Pfam accession number PF02275).

Human AC is a heterodimer composed of 13- (α) and 40-kDa (β) subunits that are derived from a common 55-kDa precursor encoded by a single cDNA (47). NAAA of human, rat, and mouse had a sequence (CTSIVAQDS) highly homologous to the N-terminal sequence of the β-subunit of AC (CTSIVAEDK).

NAE Hydrolyzing Activities of Recombinant NAAA and AC—We overexpressed hNAAA cDNA in HEK293 cells by the Lipofect method. To detect its NAE hydrolyzing activity, we overexpressed hNAAA cDNA in HEK293 cells by the Lipofect method. In order to detect its NAE hydrolyzing activity, we used the following methods.

**Fig. 2.** NAE hydrolyzing activity of NAAA and AC expressed in HEK293 cells. A, NAE hydrolyzing activity of recombinant NAAA and AC as analyzed by TLC. The homogenates of HEK293 cells (15 μg of protein) transfected with the insert-free vector (lanes 2 and 6) or the expression vector harboring cDNA for human NAAA (lanes 3 and 7) or AC (lanes 4 and 8) or the enzyme-free buffer (lanes 1 and 5) were allowed to react with 200 μM of N-[14C]palmitoylethanolamine (lanes 1–4) or N-[14C]lauroylethanolamine (lanes 5–8) at pH 4.5 in the presence of 3 mM DTT. Nonidet P-40 (0.1%) was included in the reaction mixture (lanes 1–4) or not (lanes 5–8). The products were separated by TLC and visualized by a bioimaging analyzer. Positions of authentic compounds are indicated by arrowheads. 12:0-FA, lauric acid; 12:0-NAE, N-lauroylethanolamine; 16:0-FA, palmitic acid; 16:0-NAE, N-palmitoylethanolamine. B, reactivity of recombinant NAAA and AC with various N-acyl ethanolamines. The homogenates of HEK293 cells transfected with the insert-free vector (Mock) or the expression vector harboring human NAAA or AC were allowed to react with various [14C]NAEs (200 μM) at pH 4.5 in the presence of 3 mM DTT. Nonidet P-40 (0.1%) was included in the reaction mixture (closed columns) or not (open columns). Protein amounts used were 25 (Mock and AC) or 6 μg (NAAA). Mean values ± S.D. are shown (n = 3). 12:0, N-lauroylethanolamine; 14:0, N-myristoylethanolamine; 16:0, N-palmitoylethanolamine; 18:0, N-stearoylethanolamine; 18:1, N-oleoylethanolamine; 20:4, anandamide.
the homogenates of the transfected cells were allowed to react with \( N^{{14}} \)C-palmitoylethanolamine at pH 4.5, and the produced \( {14} \)C-palmitic acid was separated by TLC. Our previous results showed that the NAE hydrolyzing activity of rat lung NAAA was potently enhanced by the addition of DTT and non-ionic detergent Triton X-100 (40). Therefore, we measured the enzyme activity in the presence of 3 mM DTT and 0.1% Nonidet P-40, composed of a major constituent of Triton X-100. Under these conditions, the homogenates of hNAAA-transfected cells hydrolyzed \( N \)-palmitoylethanolamine with a specific enzyme activity of 8.1 ± 0.1 nmol/min/mg protein at 37 °C (Fig. 2, A, lane 3, and B). On the other hand, the homogenates of HEK293 cells transfected with the insert-free vector were almost inactive (less than 0.03 nmol/min/mg protein) (Fig. 2, A, lane 2, and B). The rat and mouse homologues (rNAAA and mNAAA) were also expressed in HEK293 cells by the same method, and the homogenates of the transfected cells exhibited the \( N \)-palmitoylethanolamine hydrolyzing activities.

When we examined substrate specificity using various NAEs with different long-chain fatty acids, \( N \)-palmitoylethanolamine was found to be the most reactive substrate (Fig. 2B). Oleamide (34) and 2-arachidonoylglycerol (48) were reported to be good substrates of FAAH. However, the homogenates of hNAAA-transfected cells hydrolyzed oleamide only at a low rate (4% of the \( N \)-palmitoylethanolamine hydrolysis) and were almost inactive with 2-arachidonoylglycerol. These catalytic properties of the recombinant NAAA were consistent with previous results with rat lung NAAA (40).

In relation to the above-mentioned considerable homology in the amino acid sequences between NAAA and AC, it was interesting to investigate whether or not AC also has NAE hydrolyzing activity. We prepared cDNA of human AC from CMK cells by RT-PCR, according to the published sequence (GenBank accession number U70063) (47). The sequence that we determined was completely identical with that of U70063. AC was then overexpressed in HEK293 cells by the same method as the NAAA overexpression. The results indicated that recombinant AC has relatively low but significant NAE hydrolyzing activities (Fig. 2, A, lane 8, and B). Unlike NAAA, which hydrolyzed \( N \)-palmitoylethanolamine at the highest rate, AC preferred \( N \)-lauroylethanolamine to other \( N \)-acyl ethanolamines, including anandamide and \( N \)-palmitoylethanolamine. Most interestingly, in contrast to NAAA, \( N \)-lauroylethanolamine hydrolyzing activity of AC was decreased by the addition of 0.1% Nonidet P-40 (Fig. 2B).

We also compared the effects of DTT on the NAE hydrolyzing activities between NAAA and AC. As shown in Fig. 3A, the addition of DTT in a range of 0.01 to 10 mM dose-dependently increased the \( N \)-palmitoylethanolamine hydrolyzing activity of recombinant NAAA up to 6.9-fold. In contrast, DTT showed only a weak stimulatory effect (up to 1.4-fold) on the \( N \)-lauroylethanolamine hydrolyzing activity of AC. These results indicated that both NAAA and AC have NAE hydrolyzing activity but were distinct in the substrate specificity and the effects of Nonidet P-40 and DTT. Previously, native NAAA (39, 40) and AC (52) were shown to have optimal pH at 5 and 3.8–4.3, respectively. When the pH value in the reaction mixture was changed between 3 and 11, both NAE hydrolyzing activities of recombinant NAAA and AC were the highest at pH 4.5 and hardly detectable above pH 8 (Fig. 3B). We also performed kinetic analyses on the NAE hydrolysis of these two enzymes. Both NAAA (with \( N \)-palmitoylethanolamine as substrate) and AC (with \( N \)-lauroylethanolamine) displayed typical Michaelis-Menten kinetics, with apparent \( K_m \) values of 97 and 77 μM, respectively (Fig. 4).

\( \text{Cumene Hydrolyzing Activity of Recombinant NAAA—Although Hong et al. (46) reported that acid ceramidase-like protein did not show ceramide hydrolyzing activity, we examined whether recombinant NAAA hydrolyzed ceramide under our assay conditions with 3 mM DTT, 0.1% Nonidet P-40, 0.05% BSA, and 150 mM NaCl in the reaction mixture. As a positive control, the homogenates of the HEK293 cells overexpressing recombinant AC were allowed to react with \( N^{{14}} \)C-ceramide hydrolyzing activity of AC was decreased by the addition of 0.1% Nonidet P-40 (Fig. 2B).}

\( \text{We also compared the effects of DTT on the NAE hydrolyzing activities between NAAA and AC. As shown in Fig. 3A, the addition of DTT in a range of 0.01 to 10 mM dose-dependently increased the \( N \)-palmitoylethanolamine hydrolyzing activity of recombinant NAAA up to 6.9-fold. In contrast, DTT showed only a weak stimulatory effect (up to 1.4-fold) on the \( N \)-lauroylethanolamine hydrolyzing activity of AC. These results indicated that both NAAA and AC have NAE hydrolyzing activity but were distinct in the substrate specificity and the effects of Nonidet P-40 and DTT. Previously, native NAAA (39, 40) and AC (52) were shown to have optimal pH at 5 and 3.8–4.3, respectively. When the pH value in the reaction mixture was changed between 3 and 11, both NAE hydrolyzing activities of recombinant NAAA and AC were the highest at pH 4.5 and hardly detectable above pH 8 (Fig. 3B). We also performed kinetic analyses on the NAE hydrolysis of these two enzymes. Both NAAA (with \( N \)-palmitoylethanolamine as substrate) and AC (with \( N \)-lauroylethanolamine) displayed typical Michaelis-Menten kinetics, with apparent \( K_m \) values of 97 and 77 μM, respectively (Fig. 4).}

\( \text{Ceramide Hydrolyzing Activity of Recombinant NAAA—Although Hong et al. (46) reported that acid ceramidase-like protein did not show ceramide hydrolyzing activity, we examined whether recombinant NAAA hydrolyzed ceramide under our assay conditions with 3 mM DTT, 0.1% Nonidet P-40, 0.05% BSA, and 150 mM NaCl in the reaction mixture. As a positive control, the homogenates of the HEK293 cells overexpressing recombinant AC were allowed to react with \( N^{{14}} \)C-}

\( \text{Ceramide Hydrolyzing Activity of Recombinant NAAA—Although Hong et al. (46) reported that acid ceramidase-like protein did not show ceramide hydrolyzing activity, we examined whether recombinant NAAA hydrolyzed ceramide under our assay conditions with 3 mM DTT, 0.1% Nonidet P-40, 0.05% BSA, and 150 mM NaCl in the reaction mixture. As a positive control, the homogenates of the HEK293 cells overexpressing recombinant AC were allowed to react with \( N^{{14}} \)C-ceramide at pH 4.5, and the product \( ^{{14}} \)C-ceramide was separated by TLC. The result showed the generation of ceramide acid with a specific activity of 0.43 nmol/min/mg protein at 37 °C (Fig. 5, A, lane 4, and B). Consistent with the previous report (52) revealing that human urine AC shows the highest reactivity with \( N \)-lauroylphosphoglycerol among ceramides with different N-acyl groups, recombinant AC hydrolyzed \( N^{{14}} \)C-palmitoylphosphoglycerol (C16-ceramide) at a lower rate (0.095 nmol/min/mg protein) (Fig. 5B). The homogenates of the
HEK293 cells transfected with the insert-free vector showed an endogenous ceramide hydrolyzing activity (0.014 ± 0.002 and 0.002 ± 0.002 nmol/min/mg protein) toward N-laurylethanolamine and N-palmitoylsphingosine, respectively (Fig. 5, A, lane 2 and B). When the homogenates of the cells transfected with hNAAA were allowed to react with these two ceramides, the enzyme showed low but significant ceramide hydrolyzing activities (0.003 ± 0.004 and 0.014 ± 0.002 nmol/min/mg protein, respectively) (Fig. 5, A, lane 3 and B).

Glycosylation and Processing of NAAA—To analyze possible glycosylation and post-translational processing of NAAA, the C-terminally hexahistidine-tagged human NAAA was overexpressed in HEK293 cells. Upon Western blotting of cell homogenates with anti-hexahistidine antibody, a singlet band around 52 kDa and a doublet band around 39–42 kDa were detected (Fig. 6A). The cell homogenates of HEK293 cells transfected with insert-free vector gave no detectable bands. When HEK293 cells were allowed to react with 100 μg N-[14C]laurylethanolamine or AC in the presence of 0.1% Nonidet P-40, the products were separated by TLC and visualized by a bioimaging analyzer. Positions of authentic compounds are indicated by arrowheads. 12:0-FA, lauric acid; C12-ceramide, N-laurylethanolamine. B, reactivity of recombinant NAAA and AC with N-[14C]lauroylethanolamine and N-[14C]palmitoylsphingosine. The homogenates of HEK293 cells (100 μg of protein) transfected with the insert-free vector (Mock) or the expression vector harboring human NAAA or AC were allowed to react with 100 μg of N-[14C]laurylethanolamine (C12) and N-[14C]palmitoylsphingosine (C16) in the presence of 0.1% Nonidet P-40. Mean values ± S.D. are shown (n = 3).

Because the N-terminal sequence of NAAA purified from rat lung began with Cys131 (Fig. 1A), it was suggested that rat lung NAAA is proteolytically cleaved at this position. Therefore, it was likely that recombinant human NAAA expressed in HEK293 cells was also subjected to proteolytic cleavage at the corresponding position, resulting in the generation of a 29.7-kDa peptide backbone including the tag and spacer. However, the molecular mass of the unglycosylated form (39–42 kDa) observed in Fig. 6A suggested that such a cleavage did not occur and, if any, only a short peptide (1–4 kb) was removed from the full-length NAAA (43.4 kDa). We then examined whether the recombinant NAAA could be cleaved in a cell-free system. The hexahistidine-tagged NAAA partially purified from HEK293 cells (a specific enzyme activity, 104 nmol/min/mg protein) was used for this purpose. As analyzed by Western blotting with anti-hexahistidine antibody, NAAA in this partially purified enzyme preparation existed dominantly used in this experiment was calculated to be 43.4 kDa. Thus, in this expression system NAAA appeared to be expressed as both glycosylated (52 kDa) and unglycosylated forms (39–42 kDa). Most interestingly, the tunicamycin treatment caused a considerable decrease in specific enzyme activity (Fig. 6B), suggesting that glycosylation is necessary for the full enzyme activity.

FIG. 5. Ceramide hydrolyzing activity of NAAA and AC expressed in HEK293 cells. A, ceramide hydrolyzing activity of recombinant NAAA and AC as analyzed by TLC. The homogenates of HEK293 cells (100 μg of protein) transfected with the insert-free vector (lane 2) or the expression vector harboring cDNA for human NAAA (lane 3) or AC (lane 4) or the enzyme-free buffer (lane 1) were allowed to react with 100 μg N-[14C]laurylethanolamine in the presence of 0.1% Nonidet P-40. The products were separated by TLC and visualized by a bioimaging analyzer. Positions of authentic compounds are indicated by arrowheads. 12:0-FA, lauric acid; C12-ceramide, N-laurylethanolamine. B, reactivity of recombinant NAAA and AC with N-[14C]lauroylethanolamine and N-[14C]palmitoylsphingosine. The homogenates of HEK293 cells (100 μg of protein) transfected with the insert-free vector (Mock) or the expression vector harboring human NAAA or AC were allowed to react with 100 μg of N-[14C]laurylethanolamine (C12) and N-[14C]palmitoylsphingosine (C16) in the presence of 0.1% Nonidet P-40. Mean values ± S.D. are shown (n = 3).

FIG. 4. Dependence of NAE hydrolyzing activity of NAAA and AC on substrate concentrations. The homogenates of HEK293 cells overexpressing human NAAA (closed circles, 6 μg of protein) or AC (open circles, 15 μg of protein) were allowed to react with different concentrations of N-[14C]palmitoylsphingosine (NAAA) or N-[14C]lauroylethanolamine (AC) at pH 4.5 in the presence of 3 mM DTT. Untransformed plots (A) and Lineweaver-Burk (double-reciprocal) plots (B for NAAA and C for AC) are shown. Mean values ± S.D. are shown (n = 3).
as the 52-kDa glycosylated form with a faint band at ~33 kDa (Fig. 6C, lane a). The unglycosylated band at 39–42 kDa found in Fig. 6A was hardly detectable because this form of NAAA was not solubilized from the 12,000 x g pellet by freezing and thawing. When the sample was incubated at pH 4.5 for 60 min, the 52-kDa form time-dependently decreased, whereas the 33-kDa form increased, suggesting that the 52-kDa form was converted to the 33-kDa form (Fig. 6C, lanes a–c). However, this conversion was hardly observed at pH 7.4 (Fig. 6C, lane d).

Similar analyses were performed with the enzyme treated with PNGase F (an endoglycosidase cleaving all asparagine-linked oligosaccharides). Before the incubation at pH 4.5, a major band at ~40 kDa and a minor band at ~30 kDa were detected (Fig. 6C, lane e). The former band was then time-dependently converted to the latter band at pH 4.5 but not at pH 7.4 (Fig. 6C, lanes e–h). Therefore, these two bands were presumed to be the unglycosylated forms of the 52- and 33-kDa band, respectively. The molecular mass of the processed form (~30 kDa) suggested that this cleavage occurred at or near Cys131, because the molecular mass of the resultant peptide (including a hexahistidine tag and a spacer) was calculated to be 29.7 kDa as mentioned above. This cleavage by acid treatment, however, did not cause an obvious change in specific enzyme activity (Fig. 6D). In this assay, the enzyme reaction was performed only for 5 min. During the 5-min incubation, the proteolytic cleavage did not proceed significantly (data not shown). These results revealed that a specific proteolytic cleavage of recombinant NAAA occurred at acidic pH, but this processing did not appear to be involved in the regulation of the enzyme activity.

**Intracellular Localization of NAAA**—We determined the intracellular localization of human NAAA expressed in HEK293 cells using an NAAA-GFP fusion construct. By fluorescence microscopy, the expression of the fusion protein was associated with vesicular elements in the cytoplasm (Fig. 7A). In the tunicamycin-treated cells, NAAA-GFP fusion proteins were retained in the perinuclear Golgi region. Scale bars, 10 μm.
in HEK293 cells, URB597 (63) at 3 nM (4:1, v/v) at 37 °C for 30 min. In order to inhibit endogenous FAAH, the cells were incubated with 25 μM N-[14C]palmitoylethanolamine (5000 cpm) at 37 °C for 30 min. After the reaction was terminated, radioactive degradation products were analyzed by TLC. Mean values ± S.D. are shown (n = 3).

In order to clarify whether NAAA in lysosomes can actually utilize NAEs, we examined the capability of the intact HEK293 cells overexpressing NAAA to degrade N-[14C]palmitoylethanolamine. As shown in Fig. 8, NAAA-expressing cells degraded N-palmitoylethanolamine much faster than control cells, and this degradation was inhibited by the addition of N-cyclohexanecarbonylpentadecylamine, a specific NAAA inhibitor (42). These results suggested the presence of passive or active transport of NAEs to lysosomes.

Organ Distribution of NAAA mRNA—The organ distribution of NAAA mRNA was investigated in the rat by RT-PCR (Fig. 9). NAAA mRNA was widely distributed among the various organs tested. The highest expression was observed in lung, followed by several organs including thymus, spleen, colon, and cecum. This distribution was in good agreement with that of FAAH, which was abundant in liver, brain, testis, and small intestine of rats (30).

**DISCUSSION**

Considering the biological actions of various NAEs, including anandamide (9, 53) and N-palmitoylethanolamine (1, 11, 12), it is important to understand the metabolism of NAEs in detail. Intracellular hydrolysis of NAEs has been observed in a variety of mammalian organs and cell lines and has been attributed mostly to the catalysis by FAAH (30–33). Analysis of FAAH-deficient mice revealed that this enzyme plays a crucial role for the degradation of anandamide and other NAEs in the brain (36–38). Alternatively, we previously found a second NAE-hydrolyzing amidohydrolase, referred to as NAAA, here, in CMK cells and various rat tissues such as lung, spleen, and macrophages (39, 40). The activity of this enzyme was the highest around pH 5 and was stimulated by DTT and Triton X-100. NAAA was most active with N-palmitoylethanolamine among the various NAEs tested. These catalytic properties were in contrast to those of FAAH which is most active at pH 8.5–10, is insensitive to DTT, and prefers anandamide to N-palmitoylethanolamine as substrate (39). Furthermore, NAAA was much less sensitive to phenylmethylsulfonyl fluoride and methyl arachidonoyl fluorophosphonate, which are potent FAAH inhibitors (39).

Here we determined the N-terminal sequence of NAAA purified from rat lung, and for the first time we identified the cDNAs encoding NAAA from human, rat, and mouse. With human NAAA overexpressed in HEK293 cells, we examined the catalytic properties of the recombinant enzyme. The enzyme was activated by DTT (Fig. 3A) and Nonidet P-40 (essentially the same as Triton X-100) (Fig. 2B) and showed by far the highest activity with N-palmitoylethanolamine among the tested NAEs (Fig. 2B). Its optimal pH was 4.5 (Fig. 3B). These catalytic properties agreed well with those of the native enzyme, confirming the authenticity of NAAA cDNA. Thus, it was finally demonstrated that NAAA is the second NAE-hydrolyzing enzyme, catalytically and structurally distinguishable from FAAH.

Consistent with the catalytic differences between NAAA and FAAH, cDNA cloning of NAAA in the present study revealed no homology between the amino acid sequences of these two enzymes. FAAH was reported to have not only an amidohydrolase activity but also a high esterase activity for fatty aeyl esters such as 2-arachidonoylglycerol (48) and methyl arachidonate (54), whereas rat lung NAAA (40) and recombinant NAAA (the present study) were almost inactive with ester compounds. Based on these differences in the structure and function between the two enzymes, the development of specific inhibitors of NAAA without acting on FAAH should be promising. Indeed, we have recently reported some ester and amide compounds structurally related to N-palmitoylethanolamine, such as N-cyclohexanecarbonylpentadecylamine, to be specific NAAA inhibitors without inhibitory effects on FAAH (41, 42). More potent and selective inhibitors will be useful to elucidate the physiological roles of NAAA.

NAAA was essentially identical to a gene product, termed acid ceramidase-like protein, the deduced amino acid sequence of which had been noted to significantly resemble AC. NAAA,
which we cloned from CMK cells, was different from acid ceramide-like protein from the human placenta cDNA library only in one amino acid residue out of 359 residues. Specifically, Leu$^{334}$ of acid ceramide-like protein was replaced by phenylalanine in NAAA. The human gene was reported to be located in the region of 4q21.1 (46). We found that NAAA belongs to the choryloxygluhydrolase family (Pfam PF02275), which includes AC, choryloxygluhydrolase (conjugated bile acid hydrodrolase), and penicillin V acylase (55, 56). These enzymes cleave carbon-nitrogen bonds, other than peptide bonds, in linear amides.

Human AC is a lysosomal enzyme that hydrolyzes ceramide to sphingosine and free fatty acid with an optimal pH at 3.8–4.3 (52, 57). The mature form of AC is a heterodimeric glyco-protein that is composed of unglycosylated α-subunit (molecular mass, ~13 kDa) and glycosylated β-subunit (~40 kDa; peptide backbone, 28 kDa) derived from its single precursor polypeptide (52). Considering the similarity of the primary structure of NAAA to that of AC, it was likely that NAAA is also subjected to glycosylation and proteolytic cleavage during maturation. With the aid of tunicamycin and PNGase F, we could show that the recombinant NAAA is N-glycosylated (Fig. 6). In accordance with this finding, the deduced primary structure of human NAAA had six potential N-glycosylation sites (Asn-Xaa-(Ser/Thr)). The glycosylation appeared to be necessary for the full enzyme activity. The proteolytic cleavage of NAAA was strongly suggested because the N-terminal amino acid residue of the rat lung NAAA was determined to be Cys$^{131}$ (Fig. 1). Most interestingly, Cys$^{131}$ corresponded with Cys$^{143}$ of human AC, which is the N-terminal residue of the β-subunit. The cleavage of NAAA was then confirmed by Western blotting with the C-terminally hexahistidine-tagged NAAA, which was specifically cleaved at pH 4.5, but not at pH 7.4, in a cell-free system (Fig. 6). Judging from the molecular mass of the cleaved form, the cleavage appeared to occur at or near Cys$^{131}$. In the case of AC, the mature form comprises an αβ-heterodimer through disulfide bond(s) (52). However, between the SDS-PAGE under reducing conditions and that under non-reducing conditions, the electrophoretic mobility of the cleaved form of NAAA did not change (data not shown), suggesting that unlike AC, NAAA does not form a heterodimer connected through disulfide bond(s).

Some lysosomal enzymes such as cathepsin L (58) and tripeptidyl-peptidase I (59) were also reported to be subjected to in vitro processing under acidic conditions and activated by the cleavage. In contrast, our results suggested that the cleavage of NAAA hardly affected the catalytic activity. Therefore, further analyses will be necessary to clarify the physiological significance of the cleavage of NAAA, which may include the alteration of stability and intracellular localization of the enzyme. Moreover, the cleavages of cathepsin L and tripeptidyl-peptidase I were self-catalyzed (59, 60), raising the possibility that NAAA is also cleaved self-catalytically.

We showed that the organ distribution of NAAA mRNA in rats was similar to that of the enzyme activity reported previously (40), which was the highest in lung, followed by spleen, small intestine, thymus, and ecum. However, in human tissues the mRNA of acid ceramide-like protein was shown to be widely distributed with higher expression in the liver and kidney (46). Thus, the organ distribution of NAAA appeared to be different between rat and human. In addition, the distribution of rat NAAA was considerably different from that of rat FAAH, suggesting distinct physiological roles between these two enzymes. As to the intracellular localization, NAAA-GFP fusion protein showed lysosome-like distribution in HEK293 cells (Fig. 7). This observation was consistent with the previous finding (46) that GFP fusion protein of acid ceramide-like protein was present in a punctate pattern located throughout the cytoplasm of the COS-1 cells. The localization in lysosomes was also supported by the optimal pH of NAAA at 4.5. Tunicamycin treatment caused the retention of the enzyme in Golgi-like compartments. This retention could result from the failure of the recognition of the enzyme by mannose 6-phosphate receptor. By analogy with the ceramide accumulation in Farber disease, the inherited deficiency of AC (47, 61), it will be of interest to examine whether deficiency of NAAA might lead to accumulation of NAEs.

One of the intriguing findings in the present study is that AC hydrolyzed not only ceramide but also NAE with an optimal pH at 4.5. We also found that NAAA possessed a low but measurable ceramide hydrolyzing activity in addition to the NAE hydrolyzing activity. Thus, NAAA and AC shared the catalytic activities hydrolyzing the amide bonds of NAE and ceramide. However, several differences in the NAE hydrolyzing activity were observed as follows. First, the substrate specificity with respect to the N-acyl groups of NAEs was different; NAAA was by far the most active with N-palmitoylethanolamine followed by N-myristoylethanolamine, whereas AC hydrolyzed N-lau roylethanolamine at the highest rate with very low activity toward bioactive NAEs, including N-palmitoylethanolamine and anandamide. Most notably, the selectivity of AC for N-lau roylethanolamine corresponds to the observation that the ceramide hydrolyzing activity of AC was the highest with N-lau roylethanolamine among the various N-acylsphingosines (52). Second, DTT stimulated the NAE hydrolyzing activity of AC only weakly. Third, Nonidet P-40 caused inhibition of the N-lau roylethanolamine hydrolysis by AC. As described above, DTT and Nonidet P-40 acted as potent stimulators for NAAA. Most interestingly, N-oleylethanolamine was earlier reported to inhibit ceramidase (62), which might be related to the NAE hydrolyzing activity of AC.

In conclusion, we identified the eDNA of NAAA and confirmed it as the second NAE-hydrolyzing enzyme. NAAA was revealed to be a glycoprotein localizing mainly in lysosomes. Its preference of N-palmitoylethanolamine as substrate suggests the physiological importance of NAAA in the regulation of this compound. Our studies also revealed the structural and functional resemblance between NAAA and AC.

REFERENCES
1. Schmid, H. H. O., Schmid, P. C., and Natarajan, V. (1990) Prog. Lipid Res. 29, 1–43
2. Hansen, H. S., Moesgaard, B., Hansen, H. H., and Petersen, G. (2000) Chem. Phys. Lipids 108, 135–150
3. Schmidt, H. H. O., and Berdyshhev, E. V. (2002) Prostaglandins Leukotrienes Essent. Fatty Acids 66, 363–376
4. Sugiuira, T., Kobayashi, Y., Oka, S., and Waku, K. (2002) Prostaglandins Leukotrienes Essent. Fatty Acids 66, 173–192
5. Bachur, N. R., Masek, K., Melmon, K. L., and Udenfriend, S. (1965) J. Biol. Chem. 240, 1019–1024
6. Epps, D. E., Schmid, P. C., Natarajan, V., and Schmid, H. H. O. (1979) Biochim. Biophys. Res. Commun. 90, 628–633
7. Devane, W. A., Hanus, L., Breuer, A., Pertwee, R. G., Stevenson, L. A., Griffin, G., Gibson, D., Mandelbaum, A., Etinger, A., and Mechoulam, R. (1992) Science 258, 1946–1949
8. Hanus, L., Gopher, A., Almog, S., and Mechoulam, R. (1993) J. Med. Chem. 36, 3932–3934
9. Di Marzo, V. (1996) Biochim. Biophys. Acta 1392, 153–175
10. van der Stelt, M., and Di Marzo, V. (2004) Eur. J. Pharmacol. 271, 1827–1834
11. Lambert, D. M., and Di Marzo, V. (1999) Curr. Med. Chem. 6, 757–773
12. Lambert, D. M., Vandevoorde, S., Jonsson, K. O., and Fowler, C. J. (2002) Curr. Med. Chem. 9, 683–694
13. Facci, L., Dal Toso, R., Romanello, S., Buriani, A., Skaper, S. D., and Leon, A. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 3376–3380
14. Mazzari, S., Canella, R., Petrelli, L., Marcolongo, G., and Leon, A. (1996) Eur. J. Pharmacol. 300, 227–236
15. Berdyshhev, E., Boichat, E., Corbel, M., Germain, N., and Lagente, V. (1998) Life Sci. 63, PL125–PL129
16. Calignano, A., La Rana, G., Giuffrida, A., and Piomelli, D. (1998) Nature 394, 277–281
17. Calignano, A., La Rana, G., and Piomelli, D. (2001) Eur. J. Pharmacol. 419, 191–198
Molecular Characterization of N-Acylethanolamine-hydrolyzing Acid Amidase, a Novel Member of the Choloylglycine Hydrolase Family with Structural and Functional Similarity to Acid Ceramidase
Kazuhiro Tsuboi, Yong-Xin Sun, Yasuo Okamoto, Nobukazu Araki, Takeharu Tonai and Natsuo Ueda

J. Biol. Chem. 2005, 280:11082-11092.
doi: 10.1074/jbc.M413473200 originally published online January 17, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M413473200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 63 references, 20 of which can be accessed free at http://www.jbc.org/content/280/12/11082.full.html#ref-list-1