A Second Fatty Acid Amide Hydrolase with Variable Distribution among Placental Mammals*

Received for publication, July 13, 2006, and in revised form, September 15, 2006. Published, JBC Papers in Press, October 2, 2006, DOI 10.1074/jbc.M600664200

Binqing Q. Wei†, Tarjei S. Mikkelsen†, Michele K. McKinney‡, Eric S. Lander§, and Benjamin F. Cravatt†1

From 1The Skaggs Institute for Chemical Biology and Departments of Cell Biology and Chemistry, The Scripps Research Institute, La Jolla, California 92037 and 2The Broad Institute of MIT and Harvard, Cambridge, Massachusetts 02142

Fatty acid amides constitute a large and diverse class of lipid transmitters that includes the endogenous cannabinoid anandamide and the sleep-inducing substance oleamide. The magnitude and duration of fatty acid amide signaling are controlled by enzymatic hydrolysis in vivo. Fatty acid amide hydrolase (FAAH) activity in mammals has been primarily attributed to a single integral membrane enzyme of the amidase signature (AS) family. Here, we report the functional proteomic discovery of a second membrane-associated AS enzyme in humans that displays FAAH activity. The gene that encodes this second FAAH enzyme was found in multiple primate genomes, marsupials, and more distantly related vertebrates, but, remarkably, not in a number of lower placental mammals, including mouse and rat. The two human FAAH enzymes, which share 20% sequence identity and are referred to hereafter as FAAH-1 and FAAH-2, hydrolyzed primary fatty acid amide substrates (e.g. oleamide) at equivalent rates, whereas FAAH-1 exhibited much greater activity with N-acyl ethanolamines (e.g. anandamide) and N-acyl taurines. Both enzymes were sensitive to the principal classes of FAAH inhibitors synthesized to date, including O-aryl carbamates and α-keto heterocycles. These data coupled with the overlapping, but distinct tissue distributions of FAAH-1 and FAAH-2 suggest that these proteins may collaborate to control fatty acid amide catabolism in primates. The apparent loss of the FAAH-2 gene in some lower mammals should be taken into consideration when extrapolating genetic or pharmacological findings on the fatty acid amide signaling system across species.

The fatty acid amide family of bioactive lipids can be divided into at least three chemical classes: N-acyl ethanolamines (NAEs)2 (e.g. C20:4 NAE (anandamide), Ref. 1), fatty acid primary amides (e.g. 9Z-octadecenamide (oleamide), Ref. 2); and N-acyl amino acids (e.g. N-acyl taurines (NATs), Ref. 3). The NAE anandamide acts as an endogenous agonist for the central cannabinoid receptor (CB1) (1) and modulates several neurobehavioral processes, including pain (4), feeding (5), and memory (6). Oleamide is a sleep-inducing lipid that accumulates in the cerebrospinal fluid of sleep-deprived animals (2, 7). Oleamide has been shown to affect several protein receptors, including serotonin (8), GABA (9, 10) and cannabinoid (11) receptors, as well as gap junctions (12), and at least a subset of these proteins appears to be critical for mediating the hypnotic effects of oleamide (13, 14). NATs are representative members of a large family of N-acyl amino acids that vary in both acyl chain and amino acid content (3, 15–17). These lipids have been shown to modulate pain sensation (17) and activate TRP channels (16).

The signaling function of fatty acid amides is terminated by enzymatic hydrolysis in vivo. A principal enzyme involved in this process is fatty acid amide hydrolase (FAAH) (18–20). Mice with a targeted disruption in the FAAH gene (FAAH−/− mice) (21) or those treated with FAAH inhibitors (22, 23) are severely impaired in their ability to degrade fatty acid amides and show hypersensitivity to the pharmacological effects of these lipids. Blockade of FAAH activity also leads to highly elevated endogenous levels of fatty acid amides in the nervous system (21–23) and peripheral tissues (24) that correlate with analgesic (21, 22, 25), anxiolytic (22), and anti-inflammatory (24, 26) phenotypes. Interestingly, these animals do not exhibit the adverse responses that typically accompany global activation of the CB1 receptor, such as hypomotility, hypothermia, and catalepsy (21), suggesting that FAAH inhibitors may induce a therapeutically useful subset of the effects observed with direct cannabinoid receptor agonists.

FAAH is an integral membrane protein widely distributed in mammalian tissues that belongs to a large family of enzymes that share a highly conserved ~130 amino acid motif designated the “amidase signature” (AS) sequence (20). AS enzymes possess an unusual serine-serine-lysine catalytic triad, which functions to promote amide bond hydrolysis in a manner generally analogous to the serine-histidine-aspartic acid triad more commonly observed in serine hydrolases (27, 28). X-ray crystallography studies have revealed that FAAH possesses multiple modes for membrane binding, including a hydrophobic plateau domain for monotopic integration that resides adjacent to the active site and appears to grant the enzyme access to bilayer-embedded fatty acid amide substrates (29).

The majority of AS enzymes identified to date are bacterial or fungal in origin, with FAAH constituting the sole characterized...
A Second Human FAAH

member of this protein family in mammals. Here, we provide functional proteomic evidence for the existence of a second mammalian AS enzyme with FAAH activity. This “FAAH-2” enzyme (originally designated FLJ31204) exhibits overlapping, but distinct tissue distribution, substrate selectivity, and inhibitor sensitivity profiles compared with the original FAAH enzyme (FAAH-1). Remarkably, analysis of genome and gene expression databases revealed that the FAAH-2 gene is present in primates, as well as in a variety of distantly related vertebrates, but not in murids (mice and rats). These results suggest differences in the enzymatic complexity of fatty acid amide catalysis across mammalian species.

EXPERIMENTAL PROCEDURES

Materials—Cancer cell lines were obtained from the National Cancer Institute Developmental Therapeutics Program. 14C-Fatty acid amides were synthesized from their corresponding acid chlorides as reported previously (2, 3, 18).

Activity-based Protein Profiling Multidimensional Protein Identification Technology (ABPP-MudPIT) of Human Cancer Cell Lines—Cells were grown to 80% confluence in either RPMI 1640 medium (for the ovarian cancer line OVCAR-3), or Dulbecco’s modified Eagle’s medium (for the breast cancer line MCF-7), both of which contain 10% fetal calf serum, and were washed three times with phosphate-buffered saline before being harvested by scraping. Cell pellets were sonicated and Dounce homogenized in 50 mM Tris-HCl, pH 8 (Buffer 1) followed by centrifugation at 100,000 × g to separate soluble (supernatant) and membrane (pellet) proteomes. Membrane pellets were sonicated to homogeneity in Buffer 1. Protein concentrations were determined by the Lowry assay (Bio-Rad). Proteomes (1 mg/ml) were analyzed by ABPP-MudPIT, as described previously (30). Briefly, proteomes were treated with a biotin-conjugated fluorophosphate activity-based probe (FP-biotin, 5 μM) (31) for 2 h at 25 °C. Membrane proteomes were then solubilized with 1% Triton X-100 by rotating at 4 °C for 1 h. Enrichment of FP-labeled proteins by avidin-conjugated beads, protease digestion, and multidimensional liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis were performed as previously reported (30, 32). All mass spectrometric data were searched against the human IPI data base (version 1.4.2005) using the SEQUEST algorithm (33). Peptides were considered correct identifications if cross-correlation scores were above 1.8 (for +1 charge), 2.5 (+2), 3.5 (+3) and delta CN scores were above 0.08. As a control for nonspecific binding to the avidin beads, proteomes that had not been treated with FP-biotin were also analyzed (“no probe” controls). FP-biotin-treated and no-probe control proteomes were analyzed in triplicate and duplicate, respectively.

Generation and Transfection of Expression Constructs for FAAH-1 and FAAH-2—A full-length FAAH-2 cDNA clone was obtained from Open Biosystems (Clone ID 3210044). The cDNA sequence was subcloned into the pFLAG.CMV6 vector (Sigma) with an N-terminal Myc-His tag using EcoRI and BamHI restriction sites, into the pcdNA3.1/Myc-His vector with a C-terminal Myc-His tag using EcoRI and XbaI sites, and into the pcDNA3 vector (Invitrogen) with an N-terminal Kozak sequence (GCCACC) using EcoRI and XbaI sites. Authenticity of all constructed plasmids was confirmed by DNA sequencing. Transient transfection of COS-7 cells was performed using the FuGene transfection reagent following manufacturer’s protocols (Roche Applied Science). The inclusion of an epitope tag at the C terminus of FAAH-2 impaired the expression of active enzyme (data not shown), and therefore, an N-terminal FLAG-tagged construct was used for the characterization of recombinant FAAH-2.

ABPP of FAAH-transfected Cells—Transfected cell proteomes (1 mg/ml) were treated with a rhodamine-conjugated FP probe (FP-rhodamine (34); 100 nM, 1 h in Buffer 1 at 25 °C). A portion of the labeled samples was then treated with PNGase F (New England Biolabs) to provide deglycosylated proteomes. Reactions were quenched with an equal volume of standard 2× SDS/PAGE loading buffer (reducing) and heated for 8 min at 90 °C. Proteome samples were resolved by SDS/PAGE and blotted for 1 h. Enzymatic activity was monitored using specific binding to the avidin beads, proteome that had not been washed three times with phosphate-buffered saline before being harvested by scraping. Cell pellets were sonicated and Dounce homogenized in 50 mM Tris-HCl, pH 8 (Buffer 1) followed by centrifugation at 100,000 × g to separate soluble (supernatant) and membrane (pellet) proteomes. Membrane pellets were sonicated to homogeneity in Buffer 1. Protein concentrations were determined by the Lowry assay (Bio-Rad). Proteomes (1 mg/ml) were analyzed by ABPP-MudPIT, as described previously (30). Briefly, proteomes were treated with a biotin-conjugated fluorophosphate activity-based probe (FP-biotin, 5 μM) (31) for 2 h at 25 °C. Membrane proteomes were then solubilized with 1% Triton X-100 by rotating at 4 °C for 1 h. Enrichment of FP-labeled proteins by avidin-conjugated beads, protease digestion, and multidimensional liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis were performed as previously reported (30, 32). All mass spectrometric data were searched against the human IPI data base (version 1.4.2005) using the SEQUEST algorithm (33). Peptides were considered correct identifications if cross-correlation scores were above 1.8 (for +1 charge), 2.5 (+2), 3.5 (+3) and delta CN scores were above 0.08. As a control for nonspecific binding to the avidin beads, proteomes that had not been treated with FP-biotin were also analyzed (“no probe” controls). FP-biotin-treated and no-probe control proteomes were analyzed in triplicate and duplicate, respectively.

FAAH Substrate Assays—FAAH assays were performed by following the conversion of 14C-labeled substrates to their corresponding fatty acid using a thin layer chromatography (TLC) assay as described previously (27, 35). Reactions were conducted at 37 °C in a buffer of 125 mM Tris-HCl, 1 mM EDTA, 0.1% Triton X-100 (pH 9.0), except for pH rate profile assays, which were performed using a buffer of 50 mM Bis-Tris propane, 50 mM CAPS, 50 mM citrate, 150 mM NaCl, and 0.05% Triton X-100 (pH 6, 7, 8, or 9, adjusted using HCl or NaOH). Reactions performed in triplicate were initiated by incubating membrane lysates of COS-7 cells expressing FAAH-1 or FAAH-2 with 100 μM substrate, and were quenched with 0.5 N HCl at two time points. Fatty acids were separated from the corresponding substrates by TLC in 65% ethyl acetate/35% hexane, and the radioactive compounds were quantified using a Cyclone PhosphorImager (PerkinElmer Life Sciences). Appropriate protein concentrations and time points were chosen for each substrate-enzyme combination to keep substrate conversion between 5 and 20%. Background hydrolysis was measured using matching concentrations of the membrane lysate of COS-7 cells transfected with empty pFLAG.CMV6 vector. FAAH-1 was found to express at ~6-fold higher levels than FAAH-2 in COS-7 cells, as judged by active site titration with...
FP-rhodamine (1 μM, 1 h) following previously described protocols (36). Relative substrate hydrolysis data shown in Table 2 were normalized to account for this difference in expression.

FAAH Inhibitor Assays—Inhibitor analysis was carried out with membrane lysates using two methods. First, inhibitor potency was examined by competitive ABPP as described previously (37). Briefly, proteomes (0.01 mg/ml for FAAH-1, 0.1 mg/ml for FAAH-2) were preincubated with varying concentrations of inhibitors (1 nM to 100 μM) for 10 min at 25 °C prior to the addition of FP-rhodamine (100 nM). The reactions were then quenched after 5 min with one volume of 2× SDS loading buffer (reducing), resolved by SDS/PAGE, and visualized by in-gel fluorescence scanning. Inhibitor potency was measured as a percentage reduction in fluorescent signals relative to control samples (treated with Me2SO alone). Inhibitor potency was also analyzed by using the radioactive substrate assay described above (27, 35). Briefly, hydrolysis of [14C]oleamide by membrane proteomes was determined in the presence of varying concentrations of inhibitors (1 nm-10 μM, preincubated for 10 min) at 25 °C. Dose–response curves obtained by both methods from three independent trials at each inhibitor concentration were normalized to account for this difference in expression.

RT-PCR Analysis of Tissue Distribution of FAAH-1 and FAAH-2—First-strand cDNA from 12 human tissues (Origene Technologies) were used as the templates. PCR was performed according to the vendor suggested protocol using TaqDNA polymerase (New England Biolabs) for 40 cycles. Primers were designed for FAAH-1 (forward: 5'-GCACACGCTGTTCCC- TTCTTG-3'; reverse: 5'-GTTCATCCGACGCACT- TCATGTG-3') and FAAH-2 (forward: 5'-CCTGGGATCAA- AAGGTATAACTGACACAAAGG-3'; reverse: 5'-CTT- TCTTCCCAAACAGCAGCTCAATGG-3'). The provided control primers for β-actin were used.

RESULTS

Evidence for a Second AS Enzyme in Humans That Possesses FAAH Activity—In the course of conducting activity-based protein profiling (ABPP) studies on a panel of human cancer cell lines, we identified an uncharacterized AS enzyme, FLJ31204, as a target of the functional proteomics probe fluorophosphonate-biotin (FP-biotin; (31)). FP-biotin covalently labels the conserved serine nucleophile in a wide range of serine hydrolases, including AS enzymes, and has served as a powerful tool for the discovery of new members of this large and diverse enzyme class (36, 40). Using an LC-MS platform for the analysis of FP-biotin-treated proteomes referred to as ABPP-MudPIT (30), we identified FLJ31204 in the cancer lines MCF-7 and OVCAR-3 with a sequence coverage of 18–20% (Table 1). The enzyme was detected in the membrane, but not soluble fraction of cancer cells (Fig. 1) and was absent in control samples not treated with FP-biotin (data not shown). The more thoroughly characterized human AS enzyme FAAH was also identified in the membrane fractions of MCF-7 and OVCAR-3 cells (Fig. 1).

TABLE 1

<table>
<thead>
<tr>
<th>Identified tryptic peptides</th>
<th>Observed charge states</th>
<th>Cell lines</th>
</tr>
</thead>
<tbody>
<tr>
<td>K.TPRPb/TEPLLLLSGMLQAK.*</td>
<td>+2, +3</td>
<td>OVCAR-3, MCF7</td>
</tr>
<tr>
<td>K.EVDMb/LGGDGVLYPSHPTVAPK.H</td>
<td>+2, +3</td>
<td>OVCAR-3, MCF7</td>
</tr>
<tr>
<td>K.LAEQb/DEATLENKbWPGIVLPTVK.E</td>
<td>+2, +3</td>
<td>OVCAR-3, MCF7</td>
</tr>
<tr>
<td>K.EAFQ/QMPNSSGMLMR.R</td>
<td>+2</td>
<td>MCF7</td>
</tr>
<tr>
<td>K.GQPFLAVGQELFCTGPMCRY</td>
<td>+2</td>
<td>OVCAR-3</td>
</tr>
<tr>
<td>K.RKDVNPENNGVSKY</td>
<td>+2</td>
<td>MCF7</td>
</tr>
<tr>
<td>K.CIDVQOAYINJ.</td>
<td>+2</td>
<td>OVCAR-3</td>
</tr>
<tr>
<td>K.VQDQDLRTMQKK.</td>
<td>+2</td>
<td>OVCAR-3</td>
</tr>
</tbody>
</table>

* Boundaries of the tryptic peptides are marked by dots.

The assembled genomes of chimpanzee (assembly version panTro1), macaque (rheMac2), dog (canFam2), cow (bosTau2), mouse (mm8), rat (rn4), opossum (monDom1), chicken (galGal2), frog (xenTro2), zebrafish (danRer4), tetraodon (tettNid1), and fugu (fr1) downloaded from the UCSC Genome Browser; and using discontiguous Mega BLAST against the NCBI wgs data base and Trace Archive. Inferred amino acid sequences were manually edited to remove artifacts because of alignment errors or low quality sequence information, and subsequently re-aligned using ClustalW (39).

Genomic Analysis of Species Distribution of FAAH-2—Orthologs of FAAH-2 were identified using tblastn (38) against the assembled genomes of chimpanzee (assembly version panTro1), macaque (rheMac2), dog (canFam2), cow (bosTau2), mouse (mm8), rat (rn4), opossum (monDom1), chicken (galGal2), frog (xenTro2), zebrafish (danRer4), tetraodon (tettNid1), and fugu (fr1) downloaded from the UCSC Genome Browser; and using discontiguous Mega BLAST against the NCBI wgs data base and Trace Archive. Inferred amino acid sequences were manually edited to remove artifacts because of alignment errors or low quality sequence information, and subsequently re-aligned using ClustalW (39).

Evidence for a Second AS Enzyme in Humans That Possesses FAAH Activity—In the course of conducting activity-based protein profiling (ABPP) studies on a panel of human cancer cell lines, we identified an uncharacterized AS enzyme, FLJ31204, as a target of the functional proteomics probe fluorophosphonate-biotin (FP-biotin; (31)). FP-biotin covalently labels the conserved serine nucleophile in a wide range of serine hydrolases, including AS enzymes, and has served as a powerful tool for the discovery of new members of this large and diverse enzyme class (36, 40). Using an LC-MS platform for the analysis of FP-biotin-treated proteomes referred to as ABPP-MudPIT (30), we identified FLJ31204 in the cancer lines MCF-7 and OVCAR-3 with a sequence coverage of 18–20% (Table 1). The enzyme was detected in the membrane, but not soluble fraction of cancer cells (Fig. 1) and was absent in control samples not treated with FP-biotin (data not shown). The more thoroughly characterized AS enzyme FAAH was also identified in the membrane fractions of MCF-7 and OVCAR-3 cells (Fig. 1).

The FLJ31204 and FAAH proteins share ~20% sequence identity across their entire primary structures (Fig. 2). Despite this limited homology, both enzymes possess a predicted N-terminal transmembrane domain (dashed line, Fig. 2) and an AS sequence containing the serine-serine-lysine catalytic triad (solid line, Fig. 2), along with other residues important for catalysis. Thus, FLJ31204 appears to contain the structural features...
required to act as an active amidase. Based on this sequence analysis and the biochemical data presented below, we have elected to refer hereafter to FLJ31204 as FAAH-2, with the original FAAH protein being designated FAAH-1.

COS-7 cells transiently transfected with a cDNA encoding a FLAG-tagged FAAH-2 fusion protein were found to express a 60-kDa protein that reacted strongly with a rhodamine-tagged FP probe (FP-rhodamine) (Fig. 3A, upper panel). A weaker 58-kDa FP-reactive protein was also observed, which likely represents a version of FAAH-2 lacking the FLAG tag, as only the 60-kDa protein was detected with anti-FLAG antibodies (Fig. 3A, lower panel). The 58- and 60-kDa FP-reactive proteins were observed in the membrane, but not soluble fraction of FAAH-2-transfected cells and were not detected in mock-transfected cells (Fig. 3A). Treatment with PNGaseF did not alter the migration of FAAH-2, suggesting that it is not subject to N-linked glycosylation. FAAH-2-transfected cells also displayed robust oleamide hydrolase activity compared with their mock-transfected counterparts (Fig. 3B).

**FAAH-1 and FAAH-2 Display Distinct Substrate Selectivity Profiles**—FAAH-1 has been shown to catalyze the hydrolysis of three main classes of endogenous amidated lipids: fatty acid primary amides (18), NAEs (18), and NATs (3). We therefore selected representative members of these lipid classes for comparative analysis with mock-, FAAH-1-, and FAAH-2-transfected proteomes (primary amide: oleamide (C18:1); NAEs: anandamide (C20:4), N-oleoyl (C18:1), and N-palmitoyl (C16:0) ethanolamine; NAT: N-oleoyl taurine (C18:1)). Given the limited amounts of enzyme available from transfected cell systems, substrates were compared at a single concentration (100 μM) at pH 9.0, where both enzymes were found to display near-maximal activity (Fig. 4A). Data were then normalized to the amounts of active FAAH-1 and FAAH-2 in transfected cell proteomes, estimated by active site titration with FP-rhodamine, as described previously (36).

FAAH-1 and FAAH-2 showed nearly equivalent rates of hydrolysis with oleamide (Table 2). In contrast, FAAH-1 was much more active with all of the NAEs tested, hydrolyzing C18:1-NAE, C16:0-NAE, and anandamide at 3-, 11-, and 38-fold greater rates than FAAH-2. Interestingly, the two enzymes showed very distinct acyl chain selectivity profiles, with FAAH-1 preferring the polyunsaturated substrate anandamide over monounsaturated (C18:1) and saturated (C16:0) NAEs, while FAAH-2 showed greatest activity with C18:1 NAE (Fig. 4B). Only FAAH-1 hydrolyzed C18:1 NAT (Table 2). These data indicate that FAAH-1 and FAAH-2 accept a wide range of endogenous fatty acid amides as substrates, but with distinct acyl chain and head group preferences.

**FAAH-1 and FAAH-2 Share Similar Inhibitor Sensitivity Profiles**—The primary role that FAAH-1 plays in regulating endocannabinoid signaling in vivo has inspired efforts to develop inhibitors of this enzyme for basic research and medicinal purposes. Multiple classes of potent inhibitors of FAAH-1 have been generated, including carbamates...
Representative members of these inhibitor groups were tested for their relative potency against FAAH-1 and FAAH-2 using a competitive ABPP assay (37). Each of the inhibitors tested was active against both enzymes. The carbamate URB597 (22) and α-keto heterocycle OL-135 (23) showed greater potency for FAAH-2, while the carbamate URB532 (22) was more active against FAAH-1 (Table 3). Qualitatively similar data were obtained using a 14C-substrate assay, where URB597 was also found to inhibit FAAH-2 more potently than FAAH-1 (Table 3). The most potent inhibitor for both enzymes was JP104 (41), an agent that combines structural features of previously described carbamate (URB597) and α-keto heterocycle (e.g. compound 59 from Ref. 15) inhibitors.

FAAH-1 and FAAH-2 Display Opposite Orientations on Cell Membranes—Both FAAH-1 and FAAH-2 are predicted to possess single transmembrane domains at their respective N termini (Fig. 2, dashed lines). Interestingly, however, sequence analysis programs, such as PSORT predicted opposite orientations for the enzymes in cell membranes, such that the C-terminal catalytic domains of FAAH-1 and FAAH-2 would be located in the cytoplasmic and luminal compartments of the cell, respectively. To test this premise, we evaluated the protease sensitivities of FAAH-1 and FAAH-2 in the absence and presence of nonionic detergents. These protease protection assays were conducted with two independent proteases (trypsin and chymotrypsin) and detergents (Triton X-100 and digitonin). FAAH-1 was sensitive to degradation by either protease in the absence or presence of detergent (Fig. 5), consistent with a predominantly cytoplasmic orientation in the membrane. In contrast, FAAH-2 was only degraded by proteases following the addition of Triton X-100 or digitonin, indicating that this enzyme is luminally orientated in the membrane (Fig. 5). The selective protease sensitivity of the luminal protein Grp94 in the presence of these detergents confirmed the integrity of the membrane preparations under study (Fig. 5).

Evidence That FAAH-2 Is an Ancient Gene Present in Primates, but Not Murids—FAAH-1 is well-conserved throughout mammalian evolution, with murid and human orthologues of this enzyme sharing greater than 80% sequence identity (43).
To gain a deeper appreciation of the phylogenetic conservation of FAAH-2, we initially attempted to identify murid versions of this enzyme in public databases. However, no orthologues of the human FAAH-2 gene were found in either mouse or rat, with the nearest homologous gene instead being FAAH-1 in these mammals (20% sequence identity). We next searched all publicly available assembled genomes, as well as unassembled whole genome shotgun sequences, but failed to identify similar sequences from any non-primate placental mammal, with the exception of two partial matches from rabbit (GenBank™ accession numbers AAGW01246790 and AAGW01512787) and one from elephant (GenBank™ accession number AAGU01814490). In contrast, orthologs of FAAH-2 can readily be identified in other primates, such as the macaque (Macaca mulatta), as well as in more distantly related species, such as opossum (Monodelphis domestica) and frog (Xenopus tropicalis) (Fig. 6A). Putative orthologues can also be found in the chicken, pufferfish, and zebrafish genomes (data not shown). Interestingly, the human FAAH-2 gene was found to reside on the pericentromeric region of the short arm of the X chromosome (Fig. 6B), a location prone to rearrangements and loss during the evolution of placental mammals (44).

The apparent absence of FAAH-2 in murids and other lower mammals was further supported by an analysis of the NCBI expressed sequence tag (EST) data base. Whereas numerous ESTs corresponding to FAAH-1 were identified in mouse, rat, cow, human, and frog, ESTs for FAAH-2 originated exclusively from human and frog (Fig. 7A). A further comparative analysis of human FAAH-1 and FAAH-2 expression by reverse transcriptase-polymerase chain reaction (RT-PCR) revealed distinct tissue distributions for the enzymes (Fig. 7B). For example FAAH-1, but not FAAH-2 was highly expressed in brain, small intestine and testis. In contrast, FAAH-2, but not FAAH-1 was detected in heart. Both enzymes were found in kidney, liver, lung, and prostate.

**DISCUSSION**

The nearly complete loss of anandamide and oleamide hydrolase activity in tissues from FAAH(−/−) mice (21) suggests that FAAH-1 is the predominant enzyme catalyzing these reactions in murids. Consistent with this premise, only one additional AS enzyme is encoded by murid genomes, and this protein is predicted to act as part of an amidotransferase complex that transamidates mischarged Glu-tRNAGln in mitochondria (45). Like FAAH-1, this glutamine amidotransferase is conserved throughout mammalian evolution. We herein present biochemical and genetic evidence for the existence of a third AS enzyme found in primates and some distantly related vertebrates, but not murids. This primate AS enzyme has been designated FAAH-2, based on an ability to hydrolyze a wide range of endogenous fatty acid amides in vitro.

The unusual phylogenetic distribution of the FAAH-2 gene

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Rate of hydrolysis ( \text{nmol/min}\text{mg} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>oleamide (C18:1 FAPA)</td>
<td>0.06 ±0.03^c 9.7 ± 0.8 8.4 ± 0.8</td>
</tr>
<tr>
<td>N-palmitoyl ethanolamine (C16:0 NAE)</td>
<td>0.014 ±0.002 2.1 ± 0.23 0.20 ± 0.02</td>
</tr>
<tr>
<td>N-oleoyl ethanolamine (C18:1 NAE)</td>
<td>0.08 ±0.01 5.6 ± 0.5 1.9 ± 0.2</td>
</tr>
<tr>
<td>anandamide (C20:4 NAE)</td>
<td>0.06 ±0.01 17 ± 1 0.46 ± 0.04</td>
</tr>
<tr>
<td>N-oleoyl taurine (C18:1 NAT)</td>
<td>ND^d 0.75 ± 0.03 ND</td>
</tr>
</tbody>
</table>

^a Rates were measured at pH 9.0 using membrane fractions of COS-7 cells transfected with human FAAH-1-pcDNA3 or FAAH-2-pFLAG.CMV6 constructs. Net rates for FAAH-1 and FAAH-2 were obtained by subtracting the corresponding background (mock) rates from the measured rates. Net rates for FAAH-2 were multiplied by 6.3 to account for the 6.3-fold higher concentration of FAAH-1 than that of FAAH-2 in transfected cells.

^b Background hydrolysis rates were measured using membrane fractions of COS-7 cells transfected with pcDNA3 or pFLAG.CMV6 vectors. Both vectors gave the same background rates, and thus only the pFLAG.CMV6c values are listed.

^c Results represent averages ± S.D.; \( n = 3 \) experiments/group.

^d ND, not detected.
raised initial concerns that it might represent a pseudogene. However, several lines of evidence argue that FAAH-2 is a transcribed gene that is translated into an active protein product in human cells. First, the FAAH-2 protein was identified by ABPP as an endogenous constituent of multiple human cancer cell proteomes. Second, FAAH-2 expression was observed by RT-PCR and EST analysis in several human tissues, including multiple sites that lacked detectable FAAH-1 transcript (e.g. heart, ovary). Third, the recombinantly expressed FAAH-2 protein is a catalytically active enzyme.

Comparison of the enzymatic properties of FAAH-1 and FAAH-2 revealed intriguing differences. Perhaps most notably, FAAH-1 displayed much greater hydrolytic activity than FAAH-2 with the endocannabinoid anandamide (C20:4 NAE) (Table 2). This differential activity contrasted with the similar rates of hydrolysis displayed by these enzymes with oleamide and C18:1 NAE. FAAH-2 thus appears to prefer monounsaturated over polyunsaturated acyl chains, while FAAH-1 exhibits the opposite selectivity. These enzymes also differed in their accommodation of amine leaving groups, as FAAH-1, but not FAAH-2 catalyzed the hydrolysis of C18:1 NAT. These data suggest that FAAH-1 plays a primary role in the catabolism of anandamide and NATs, while both FAAH-1 and FAAH-2 could contribute to the degradation of monounsaturated lipid amides in vivo. Perhaps more provocatively, the opposite relative orientation of FAAH-1 and FAAH-2 in the membrane led to the identification of FAAH-2 as a novel enzyme.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>FAAH-1</th>
<th>FAAH-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>URB597</td>
<td>101 (84-120)</td>
<td>5.0 (4.3-5.9)</td>
</tr>
<tr>
<td>OL-135</td>
<td>505 (438-583)</td>
<td>26.3 (23.2-29.8)</td>
</tr>
<tr>
<td>URB532</td>
<td>206 (126-336)</td>
<td>13.4 (11.3-15.9)</td>
</tr>
<tr>
<td>JP104</td>
<td>283 (196-410)</td>
<td>1360 (970-1910)</td>
</tr>
<tr>
<td></td>
<td>0.38 (0.29-0.51)</td>
<td>1.0 (0.77-1.4)</td>
</tr>
</tbody>
</table>

*IC₅₀ values were determined by competitive ABPP unless specified otherwise. Data represent the average of three independent trials, with 95% confidence intervals shown in parentheses.

*In bold case are IC₅₀ values determined using a ¹⁴C-labeled oleamide substrate assay.
could also influence their respective access to specific fatty acid amide substrates in cells, especially if these lipids show preferential localization to the inner or outer leaflet of the membrane bilayer.

The different tissue distributions of FAAH-1 and FAAH-2 further suggest that each enzyme may make a unique and important contribution to fatty acid amide catabolism in vivo, depending on the anatomical site under consideration. For example, recent studies have shown that spontaneously hypertensive rats display increased expression of FAAH-1 in the heart, which in turn limits the hypotensive action of anandamide (46). It is interesting to speculate whether, in humans, FAAH-2 might regulate endocannabinoid signaling in the heart.

It is also important to note that the capacity to hydrolyze fatty acid amides is not the exclusive domain of enzymes from the AS class. Recently, Ueda and colleagues identified a distinct NAE hydrolase enriched in immune cells that resides in the lysosome and exhibits an acidic pH optimum (47). This lysosomal “acid” amidase is not an AS enzyme, but rather related to acid ceramidases. The contribution that acid amidase makes to fatty acid amide catabolism in vivo remains unknown, although the distinct inhibitor sensitivity profiles of this enzyme and FAAH-1 should allow straightforward pharmacological separation of their respective roles in living systems (48). In contrast, discerning the relative contribution that FAAH-1 and FAAH-2 make to controlling fatty acid amide signaling will likely require the development of more advanced pharmacological tools, as all of the FAAH-1-directed inhibitors tested in this study were also found to inhibit FAAH-2. From a biomedical perspective, inhibitors with dual specificity may be of value, as has proven to be the case for some drugs that target other lipid metabolic pathways (e.g. aspirin and the cyclooxygenase 1/2-prostaglandin system (49)).

Why might primates have retained two ancestral AS enzymes with FAAH activity, while murids evolved to utilize only one? The answer to this question is certainly not simple. Indeed, the distribution of FAAH-2 across the mammalian genomes sequenced to date is highly unusual. Our initial survey of complete or partially sequenced genomes suggests that FAAH-2 can also be found in rabbits and elephants, but not dogs, sheep, cows, or pigs. Based on the established phylogeny of mammals (50), this distribution would seem to mandate multiple independent losses of the ancient FAAH-2 gene during mammalian evolution. The repetitive nature of the pericentro-
meric region of the X chromosome where human FAAH-2 is located may have facilitated a high rate of deletion in related species, but this hypothesis will require further investigation of completely sequenced genomes for clarification. Regardless, these findings emphasize that certain metabolic pathways can escat in complexity from murids to higher mammals and such adaptations should be taken into consideration when attempting to extrapolate genetic or pharmacological findings across species.

Acknowledgments—We thank S. Niesen for assistance with ABPP-MudPIT studies, and the Cravatt laboratory for helpful discussions and critical reading of the manuscript.

REFERENCES

A Second Human FAAH


