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

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**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*-acylethanolamines (NAEs)[2] (*e.g.* C20:4 NAE (anandamide), Ref. 1), fatty acid primary amides (*e.g.* 9*Z*-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

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[2] The abbreviations used are: NAE, *N*-acylethanolamine; ABPP, activity-based protein profiling; AS, amidase signature; EST, expressed sequence tag; FAAH, fatty acid amide hydrolase; FP, fluorophosphonate; MudPIT, multidimensional protein identification technology; NAT, *N*-acyl taurine; STS, sequence tagged site; CAPS, 3-(cyclohexylamino)propanesulfonic acid.

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 catabolism across mammalian species.

## EXPERIMENTAL PROCEDURES

*Materials*—Cancer cell lines were obtained from the National Cancer Institute Developmental Therapeutics Program. $^{14}$C-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 fluorophosphonate 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 spectra 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 FLAG 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 (10% acrylamide) and visualized in-gel with an FMBio IIe flatbed fluorescence scanner (Hitachi). Integrated fluorescence band intensities were quantified using the manufacturer's supplied software. FAAH-2 protein was quantified by summing the intensities of the 58- and 60-kDa bands observed in transfected COS-7 cells.

*Western Blotting*—Proteome samples resolved by SDS-PAGE were transferred to a nitrocellulose membrane, blocked with 3% milk, and incubated with a monoclonal anti-FLAG M2 antibody (Sigma, 1:5000 dilution) overnight at 4 °C. To detect endoplasmic reticulum marker proteins, the membrane was incubated with a mouse monoclonal anti-KDEL antibody (Stressgen Bioreagents, 1:5000 dilution). To probe FAAH-1, the blot was incubated with rabbit anti-FAAH polyclonal antibodies (1:500 dilution) (35). Blots were then washed with Tris-buffered saline and Tween-20, incubated with the goat-anti-mouse or anti-rabbit IgG-horseradish peroxidase conjugates (Bio-Rad), and analyzed using a SuperSignal Western Pico Chemiluminescence Kit (Pierce), and x-ray film exposure.

*FAAH Substrate Assays*—FAAH assays were performed by following the conversion of $^{14}$C-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

**TABLE 1**
**Identification of a novel AS enzyme FLJ31204 (FAAH-2) in human cancer cell lines by ABPP-MudPIT**

| Identified tryptic peptides | Observed charge states | Cell lines |
|---|---|---|
| K.TPRPVTEPLLLLSGMQLAK.L[a] | +2, +3 | OVCAR-3, MCF7 |
| K.ELVDMLGDDGVFLYPSHPTVAPK.H | +2, +3 | OVCAR-3, MCF7 |
| K.LAEKQEDEATLENKWPFLGVPLTVK.E | +2, +3 | OVCAR-3, MCF7 |
| K.EAFQLQGMPNSSGLMNR.R | +2 | MCF7 |
| K.GQFPLAVGAQELFLCTGPMCR.Y | +2 | OVCAR-3 |
| R.IKDVNPMINGIVK.Y | +2 | MCF7 |
| K.CIDVVQAYINR.I | +2 | OVCAR-3 |
| K.VDQDLIMTQK.K | +2 | OVCAR-3 |

[a] Boundaries of the tryptic peptides are marked by dots.

FP-rhodamine (1 $\mu$M, 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 $\mu$M) 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 $Me_2SO$ alone). Inhibitor potency was also analyzed by using the radioactive substrate assay described above (27, 35). Briefly, hydrolysis of [$^{14}$C]oleamide by membrane proteomes was determined in the presence of varying concentrations of inhibitors (1 nM-10 $\mu$M, preincubated for 10 min) at 25 °C. Dose-response curves obtained by both methods from three independent trials at each inhibitor concentration were fit with Prism software (GraphPad) to obtain $IC_{50}$ values with 95% confidence intervals.

*Protease Protection Assays*—Transfected cells were pelleted, washed once with 10 ml of phosphate-buffered saline, and resuspended in buffer A (10 mM HEPES, pH 7.4, with 10 mM KCl, 1.5 mM $MgCl_2$, 5 mM EDTA, 5 mM EGTA, 250 mM sucrose). Cells were lysed by passage through a 21-gauge needle 20 times. The cell extract was then spun at 1000 × *g* for 5 min, and the supernatant removed. The pellet was resuspended in buffer A and recentrifuged at 100,000 × *g* for 15 min. The resulting pellet was gently resuspended in 500 $\mu$l of buffer C (10 mM HEPES, pH 7.4, with 10 mM KCl, 1.5 mM $MgCl_2$, 5 mM EDTA, 5 mM EGTA, 250 mM sucrose, 0.1 M NaCl) and incubated with shaking at 4 °C for 15 min. The resuspended membrane preparation (67.5 $\mu$l) were treated with 7.5 $\mu$l of either buffer C, 10% digitonin (Wako Chemical) in buffer C, or 10% Triton X-100 in buffer C. These solutions were incubated at 4 °C for 1 h. Each 75-$\mu$l sample was then treated with 0.75 $\mu$l 1 M $CaCl_2$, and split in thirds, and one-third of the sample was treated with 1 $\mu$l of either buffer C, trypsin (Promega, 1 $\mu$g) in buffer C, or $\alpha$-chymotrypsin (Roche Applied Science, 2 $\mu$g) in buffer C and incubated at 37 °C for 30 min. The reactions were stopped by the addition of SDS-PAGE loading buffer and heated at 90 °C for 8 min. Fractions were analyzed by SDS-PAGE and Western blotting procedures.

*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 (tetNig1), 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).

*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′-GCACACGCTGGTTCCCTTCTTG-3′; reverse: 5′-GTTTTCCAGCCGAACGAGACTTCATGTTG-3′) and FAAH-2 (forward: 5′-CCTGGGATCAAAAGGTTAAAACTAGACACAAAGG-3′; reverse: 5′-CTTTTCTTCCAACAAAGCCAGTCCAATGG-3′). The provided control primers for $\beta$-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 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. 3*A*, *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. 3*A*, *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. 3*A*). 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. 3*B*).

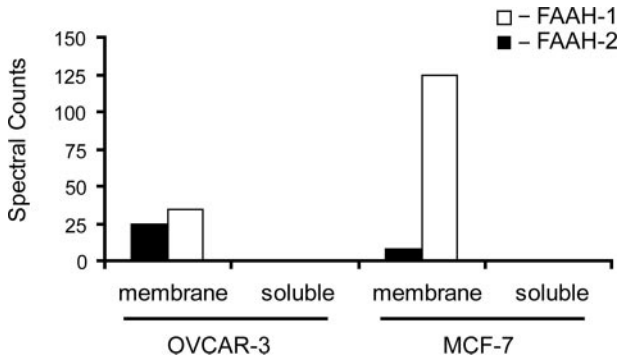



FIGURE 1. **Levels of FLJ31204 (FAAH-2) and FAAH-1 in the human cancer cell lines OVCAR-3 and MCF-7, as measured by spectral counting of peptides identified by ABPP-MudPIT (30).** FAAH-1 and FAAH-2 were identified in membrane, but not soluble proteomes from cancer cells. Neither enzyme was detected in control proteomes in which the ABPP probe FP-biotin was excluded from reactions (data not shown). Spectral counts correspond to the sum of three independent experiments per group.

*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 $\mu$M) at pH 9.0, where both enzymes were found to display near-maximal activity (Fig. 4*A*). 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. 4*B*). 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

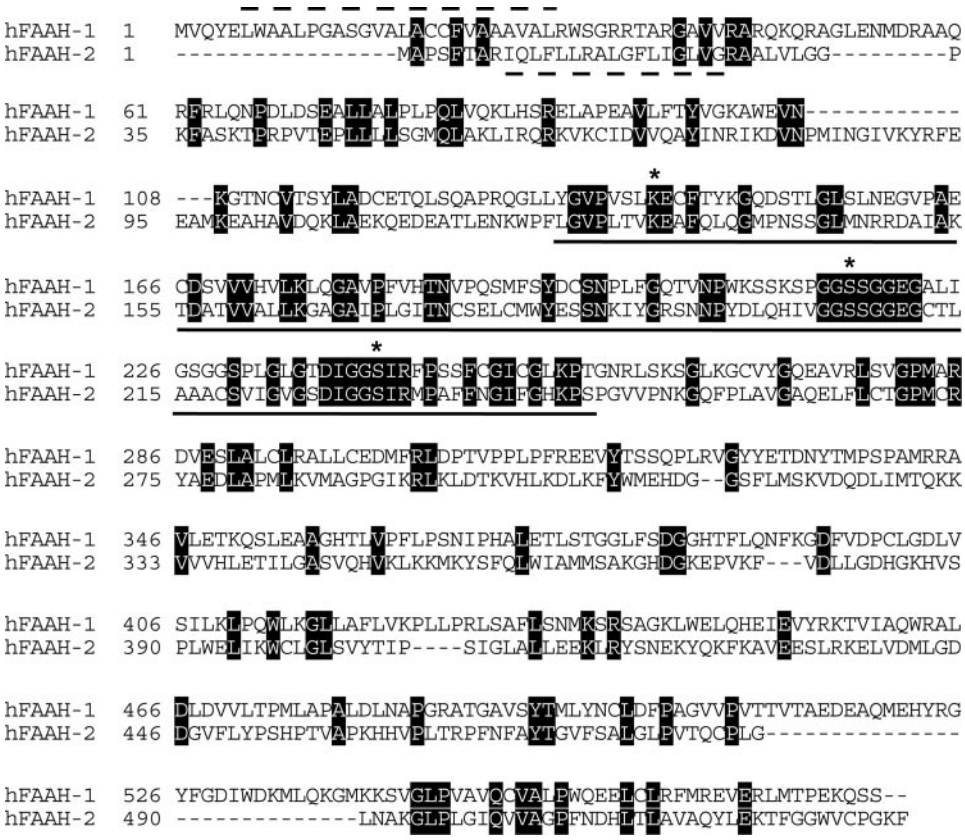



FIGURE 2. **Sequence comparison of human FAAH-1 and FAAH-2, with conserved residues shown in a black background.** The predicted N-terminal transmembrane domain of each enzyme is highlighted by a *dashed line*. The conserved AS sequence is highlighted by a *solid line*. The three residues of the serine-serine-lysine catalytic triad are shown with an *asterisk*.

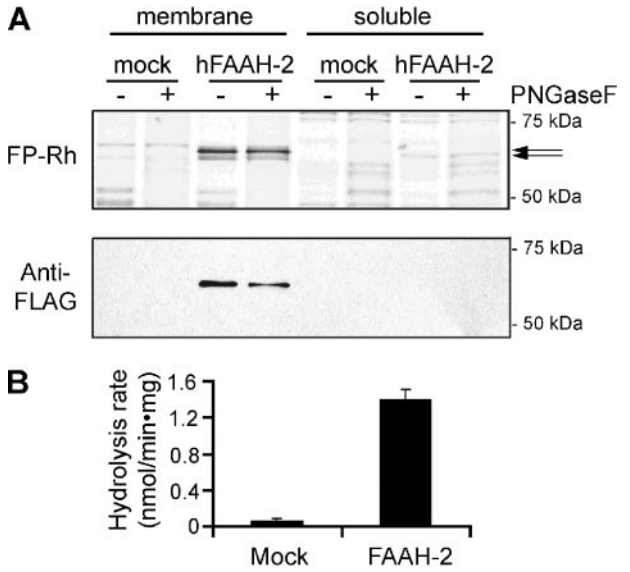



FIGURE 3. **Recombinant expression and characterization of FLAG-tagged human FAAH-2 in COS-7 cells.** *A*, membrane proteome from COS-7 cells transfected with a FLAG-tagged FAAH-2 cDNA construct exhibited FP-rhodamine (FP-Rh)-reactive proteins at 58 and 60 kDa (*arrows*, *top image* (shown in *grayscale*)). These proteins were not detected in the soluble fraction of FAAH-2-transfected cells, or in mock-transfected cells. Western blotting with anti-FLAG antibodies detected the 60-kDa, but not 58-kDa protein (*bottom image*), suggesting that the latter protein corresponded to a FAAH-2 variant in which the N-terminal FLAG tag had been proteolytically removed. Treatment with PNGaseF did not alter the migration of the FAAH-2 protein, suggesting that it is not subject to *N*-linked glycosylation. *B*, FAAH-2-transfected COS-7 cells possessed high levels of oleamide hydrolase activity compared with mock-transfected cells.

(22, 41) and α-keto heterocycles (23, 42). 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 $^{14}$C-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

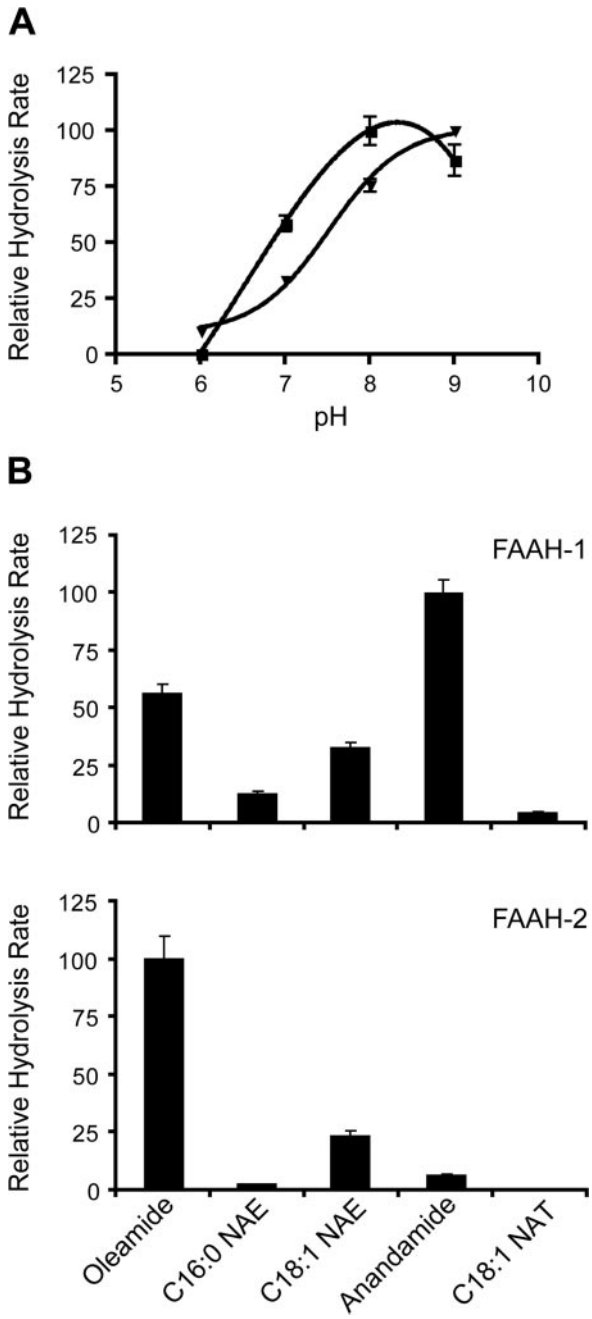
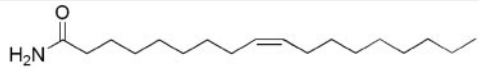
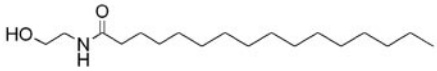
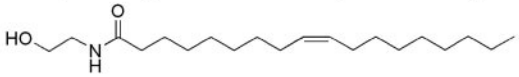
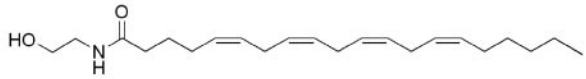
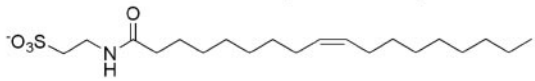



FIGURE 4. **Comparative characterization of the catalytic activities of human FAAH-1 and FAAH-2 recombinantly expressed in COS-7 cells.** *A*, pH-rate profiles of FAAH-1 (*triangles*) and FAAH-2 (*squares*) catalytic activity with the substrate oleamide. *B*, relative activity of FAAH-1 (*top graph*) and FAAH-2 (*bottom graph*) with representative fatty acid amide substrates.

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).

**TABLE 2**
**Rates of hydrolysis for FAAH-1 and FAAH-2 with representative fatty acid amide substrates**

| Substrate | Rate of hydrolysis[a] (nmol/min•mg) | | |
|---|---|---|---|
| | Mock[b] | FAAH-1 net rate | FAAH-2 net rate |
|  oleamide (C18:1 FAPA) | 0.06 ±0.03[c] | 9.7 ± 0.8 | 8.4 ± 0.8 |
|  N-palmitoyl ethanolamine (C16:0 NAE) | 0.014 ±0.002 | 2.1 ± 0.23 | 0.20 ± 0.02 |
|  N-oleoyl ethanolamine (C18:1 NAE) | 0.08 ±0.01 | 5.6 ± 0.5 | 1.9 ± 0.2 |
|  anandamide (C20:4 NAE) | 0.06 ±0.01 | 17 ± 1 | 0.46 ± 0.04 |
|  N-oleoyl taurine (C18:1 NAT) | ND[d] | 0.75 ± 0.03 | ND |

[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.

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. 6*A*). 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. 6*B*), a location prone to rearrangements and loss during the evolution of placental mammals (44).

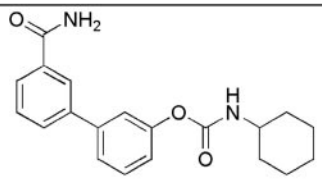
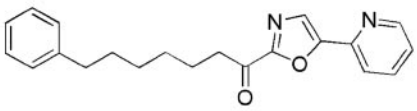
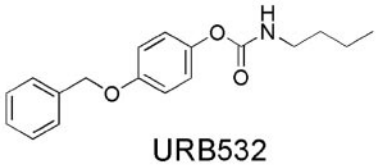
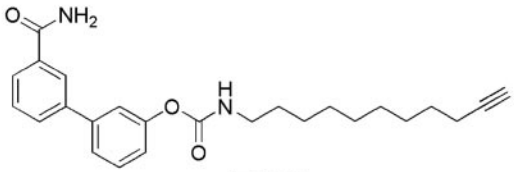
The apparent absence of FAAH-2 in murids and other lower mammals was further supported by an analysis of the NCBI expressed sequence tag (EST) data base. Whereas numerous ESTs corresponding to FAAH-1 were identified in mouse, rat, cow, human, and frog, ESTs for FAAH-2 originated exclusively from human and frog (Fig. 7*A*). 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. 7*B*). 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-tRNA$^{Gln}$ 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 3**
**$IC_{50}$ values for representative FAAH inhibitors with FAAH-1 and FAAH-2**

| Inhibitor | $IC_{50}$ (nM)[a] FAAH-1 | FAAH-2 |
|---|---|---|
|  URB597 | 101 (84-120)<br>**505 (438-583)** | 5.0 (4.3-5.9)<br>**26.3 (23.2-29.8)**[b] |
|  OL-135 | 206 (126-336) | 13.4 (11.3-15.9) |
|  URB532 | 283 (196-410) | 1360 (970-1910) |
|  JP104 | 0.38 (0.29-0.51) | 1.0 (0.77-1.4) |

[a] $IC_{50}$ values were determined by competitive ABPP unless specified otherwise. Data represent the average of three independent trials, with 95% confidence intervals shown in parentheses.
[b] In bold case are $IC_{50}$ values determined using a $^{14}C$-labeled oleamide substrate assay.

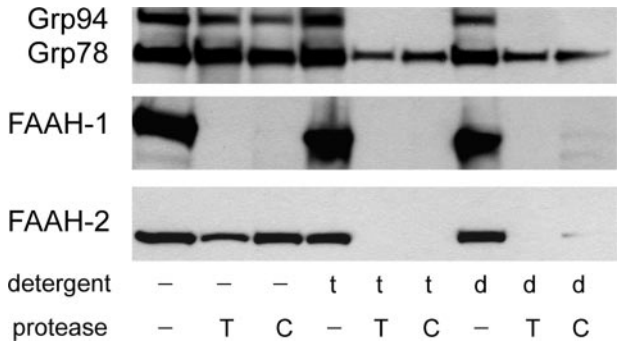



FIGURE 5. **Protease protection experiments reveal that FAAH-1 and FAAH-2 are cytoplasmically and luminally oriented membrane proteins, respectively.** Membrane fractions from COS-7 cells transfected with either the human FAAH-1 or FAAH-2 cDNA were treated with protease (trypsin (*T*) or α-chymotrypsin (*C*)), in the presence or absence of detergent (Triton X-100 (*t*) or digitonin (*d*)). All fractions were subjected to SDS-PAGE and Western blotting analysis using either anti-FAAH-1, anti-FLAG (for FAAH-2), or anti-KDEL (Grp78 and Grp94) antibodies. FAAH-1 was degraded by proteases either in the presence or absence of detergents, while FAAH-2 and Grp94 were only degraded by proteases following treatment with detergents.

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

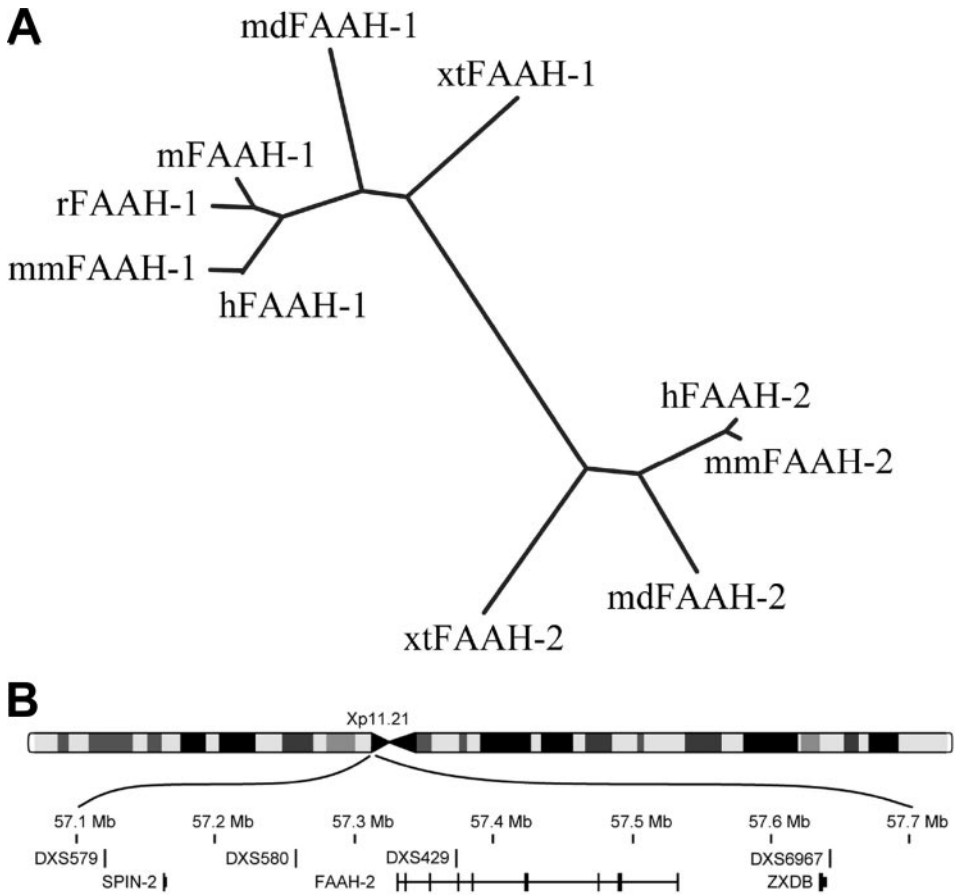



FIGURE 6. **Phylogeny and genomic location of the *FAAH-2* gene.** *A*, unrooted phylogenetic tree of FAAH-1 and FAAH-2 orthologs in human (*h*), macaque (*mm*), mouse (*m*), rat (*r*), opossum (*md*), and frog (*xt*). Branch lengths are proportional to the genetic distances between the coding sequences, as inferred by ClustalW. The tree topology was found to be robust in 1000/1000 bootstrap trials. *B*, genomic location of the human *FAAH-2* gene, with flanking genes and STS markers, as inferred from the most recent human genome assembly (hg18).

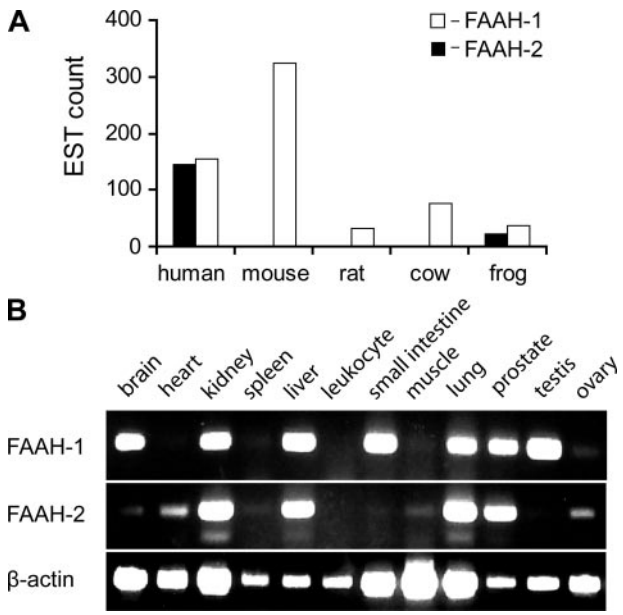



FIGURE 7. **Species and tissue distribution of FAAH-1 and FAAH-2 transcripts.** *A*, public databases contain ESTs for human, mouse, rat, cow, and frog (*Xenopus tropicalis*) FAAH-1. In contrast, for FAAH-2, only human and frog ESTs are observed. *B*, tissue distribution of human FAAH-1 and FAAH-2 as judged by RT-PCR analysis. The expression of β-actin was used as a positive control for mRNA integrity.

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 escalate 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.

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