Identification of the gene encoding alkylglycerol monooxygenase defines a third class of tetrahydrobiopterin-dependent enzymes

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Edited by Stephen J. Benkovic, Pennsylvania State University, University Park, PA, and approved June 22, 2010 (received for review February 25, 2010)

Alkylglycerol monooxygenase (glyceryl-ether monooxygenase, EC 1.14.16.5) is the only enzyme known to cleave the O-alkyl bond of ether lipids which are essential components of brain membranes, protect the eye from cataract, interfere or mediate signalling processes, and are required for spermatogenesis. Along with phenylalanine hydroxylase, tyrosine hydroxylase, tryptophan hydroxylase, and nitric oxide synthase, alkylglycerol monooxygenase is one of five known enzymatic reactions which depend on tetrahydrobiopterin. Although first described in 1964, no sequence had been assigned to this enzyme so far since it lost activity upon protein purification attempts. A functional library screen using pools of plasmids of a rat liver expression library transfected to CHO cells was also unsuccessful. We therefore selected human candidate genes by bioinformatic approaches and by proteomic analysis of partially purified enzyme and tested alkylglycerol monooxygenase activity in CHO cells transfected with expression plasmids. Transmembrane protein 195, a predicted membrane protein with unassigned function which occurs in bilateral animals, was found to encode for tetrahydrobiopterin-dependent alkylglycerol monooxygenase. This sequence assignment was confirmed by injection of transmembrane protein 195 cRNA into *Xenopus laevis* oocytes. Transmembrane protein 195 shows no sequence homology to aromatic amino acid hydroxylases or nitric oxide synthases, but contains the fatty acid hydroxylase motif. This motif is found in enzymes which contain a diiron center and which carry out hydroxylations of lipids at aliphatic carbon atoms like alkylglycerol monooxygenase. This sequence assignment suggests that alkylglycerol monooxygenase forms a distinct third group among tetrahydrobiopterin-dependent enzymes.

EC 1.14.16.5 | glyceryl-ether monooxygenase | nitric oxide synthase | phenylalanine hydroxylase | transmembrane protein 195

Tetrahydrobiopterin is a metabolite structurally related to the vitamins folic acid and riboflavin by sharing the common pterin (pyrimido[4,5-b]pyrazine) backbone. In contrast to the two vitamins which have to be taken up by the diet, however, tetrahydrobiopterin is synthesized in animals from guanosine triphosphate by the consecutive action of three enzymes (1). Five enzymatic reactions are known to depend essentially on the tetrahydrobiopterin cofactor (2). In three of these reactions, a hydroxy function is introduced into the aromatic ring of phenylalanine, tyrosine, and tryptophan by aromatic amino acid hydroxylases, which are required for the degradation of phenylalanine and for the biosynthesis of catecholamines and serotonin, important neurotransmitters and metabolism regulators. The fourth enzymatic reaction requiring tetrahydrobiopterin is catalyzed by nitric oxide synthases, which occur in three isoforms (3). After hydroxylation of the guanidino nitrogen of L-arginine in a first step, this reaction yields the radical gas nitric oxide and citrulline (4, 5). Nitric oxide synthases are required for a number of physiological processes such as blood pressure regulation, neurotransmission, and host defense against pathogens (6–8). The fifth tetrahydrobiopterin-dependent enzymatic reaction catalyzed by alkylglycerol monooxygenase (glyceryl-ether monooxygenase, EC 1.14.16.5, Fig. 1*A*) has been first described already in 1964 (9). Despite several attempts to purify and characterize this membrane bound protein (10), it still belonged to the currently 1,297 enzymes lacking sequence assignment which are called orphan enzymes (11). Alkylglycerol monooxygenase is the only enzyme known to cleave the O-alkyl ether bond in alkylglycerols, yielding an aldehyde and a glycerol derivative. The aldehyde is detoxified by conversion to the corresponding acid by fatty aldehyde dehydrogenase (EC 1.2.1.48, gene symbol ALDH3A2, Fig. S1). Tetrahydrobiopterin leaves the reaction as "quinoid" 6,7 [8H]-dihydrobiopterin (10) and is recycled to tetrahydrobiopterin by quinoid dihydropteridine reductase (Fig. S1, EC 1.5.1.34, gene symbol QDPR). The formation of 6,7[8H]-dihydrobiopterin from 4a-hydroxy-tetrahydrobiopterin, the initial enzymatic product formed from tetrahydrobiopterin, may be facilitated by 4a-carbinolamine dehydratase (EC 4.2.1.96, PCBD1) like for aromatic amino acid hydroxylases (12), but this has not yet been demonstrated for alkylglycerol monooxygenase.

Results

Protein Purification, Functional Expression Screening Attempts, and Bioinformatic Candidate Gene Selection. Although we used a robust, and sensitive assay for alkylglycerol monooxygenase activity (13), attempts to purify the protein from male rat liver, the source with the highest activity observed (10), failed due to the instability of the enzyme activity (Table S1), which could not be fully solubilized (Fig. S2). The necessity of HPLC analysis which has a capacity of about 40 assays a day precluded a full, single clone functional expression screen which would require testing of about 100,000 clones. We therefore tried an approach using plasmid pools (14). Transfection of CHO cells with 196 plasmid pools containing about 2,500 individual clones each of a rat liver

Author contributions: K.W., M.A.K., G.G., M.H., M.M., G.W.-F., and E.R.W. designed research; K.W., M.A.K., G.G., M.H., M.M., B.S., H.H.L., R.K., N.H., and E.R.W. performed research; A.H. contributed new reagents/analytic tools; K.W., M.A.K., G.G., M.H., M.M., B.S., H.H.L., G.W.-F., R.K., N.H., and E.R.W. analyzed data; and E.R.W. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1002404107/-/DCSupplemental.

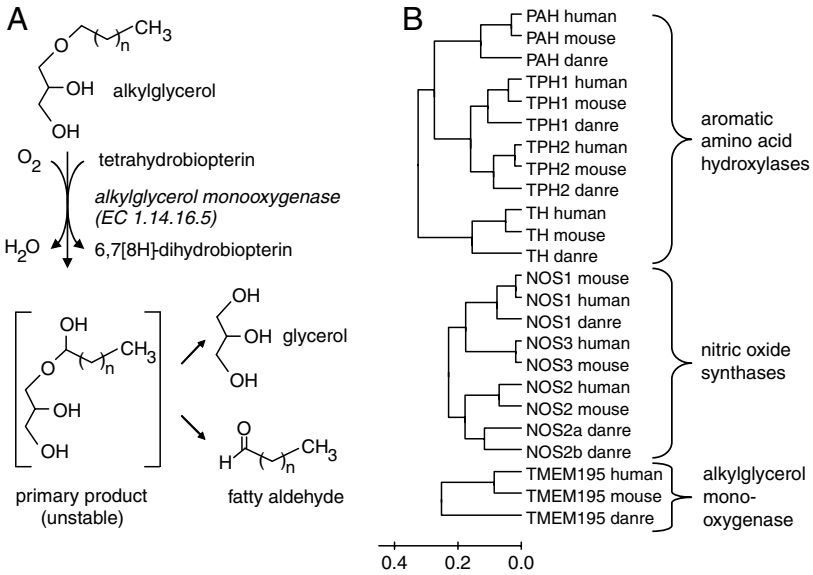

Fig. 1. Alkylglycerol monooxygenase. (*A*) The alkylglycerol monooxygenase reaction. Alkylglycerols are thought to be hydroxylated on the aliphatic carbon of the *sn*1 side chain adjacent to the ether bond in a tetrahydrobiopterin-dependent way in a mechanism sharing features with tetrahydrobiopterin-dependent enzymatic hydroxylation of aromatic amino acids. The resulting primary product, a hemiacetal, is thought to decay rapidly to the respective fatty aldehyde and glycerol (9, 10, 19). Alkylglycerol monooxygenase accepts a wide range of alkylglycerol lipids and phospholipids, and is currently the only enzyme known to cleave the O-alkyl ether bond in these compounds (see Fig. S1 for further details). Alkylglycerols with O-alkyl chain lengths of 12–20 carbon atoms are good substrates, thus *n* ranges from 10–18. (*B*) Phylogenetic tree of tetrahydrobiopterin-dependent enzymes. Protein sequences of genes for tetrahydrobiopterin-dependent enzymes from man, mouse, and zebrafish were selected from protein databases, aligned by ClustalW (Gonnet Matrix) and an UPMGA (unweighted pair group method using arithmetic averages) tree with Poisson correction drawn by MEGA4.0 using the default parameters (29). For details and accession numbers of protein sequences see Table S2. No sequence homology occurred across the three groups which differ in the form of iron required for catalysis. The scale bar gives a measure of the average fraction of amino acids exchanged between the sequences.

expression library, however, did not yield activity above background. Therefore we took advantage of the availability of the complete set of human and murine open reading frames (15) from databases which we browsed for candidates by bioinformatic and proteomic approaches, and selected the ten most promising candidates for transfection experiments (see *SI Text* for details). One candidate was selected from attempts to define a tetrahydrobiopterin binding motif common to aromatic amino acid hydroxylases and nitric oxide synthases (16), three candidates originated from a meta-structure (17) calculation screen, two candidates came from proteomic analysis of partially purified rat liver alkylglycerol monooxygenase, and four candidates, including the successful one, resulted from browsing the protein families (PFAM) database.

PFAM motifs characterize amino acid combinations in primary protein sequences which are characteristic for properties and functions of proteins (18). When browsing the 11,912 families of proteins currently defined, we realized that the fatty acid hydroxylase motif (PFAM04116) is found in proteins that catalyze hydroxylations of saturated aliphatic carbons in a way similar to alkylglycerol monooxygenase (Fig. S3), though no tetrahydrobiopterin dependence of any of these reactions had been described so far. From the human proteins containing the fatty acid hydroxylase motif, we selected three with suspected potential for undiscovered roles, sterol-C4-methyl oxidase-like (SC4MOL), chromosome 5 open reading frame 4 (C5orf4), and transmembrane protein 195 (TMEM195).

Transfection of CHO Cells with Expression Plasmids of Candidate Genes. The results of transfection of the ten selected expression plasmids of human or murine reading frames in CHO cells are shown in Fig. 2*A*. TMEM195 transfection led to an increase in tetrahydrobiopterin-dependent alkylglycerol monooxygenase activity ($p < 0.001$), all other plasmids or plasmid-free controls yielded baseline activities independent of tetrahydrobiopterin. Cotransfection of TMEM195 with ALDH3A2 (fatty aldehyde dehydrogenase) yielded a two orders of magnitude higher readout in the alkylglycerol monooxygenase assay as compared to transfection of TMEM195 alone ($p < 0.001$, Fig. 2*B*), reaching levels one order of magnitude higher than those observed in mouse tissues (Fig. 3*A*). Thus, fatty aldehyde dehydrogenase activity present in CHO cells (Fig. S4*A*) limited the amount of recombinant alkylglycerol monooxygenase activity detected with our coupled assay (13). This finding is consistent with the notion that an aldehyde (9) is the product of the TMEM195 encoded alkylglycerol monooxygenase activity (Fig. 1*A*).

Alkylglycerol monooxygenase activity generated in CHO cells by transfection of TMEM195 and ALDH3A2 displayed a Michaelis Menten constant (K_M) of 11.0 ± 1.1 μM for 1-O-pyrenedecylglycerol and 2.58 ± 0.42 μM for tetrahydrobiopterin (Fig. S5). These biochemical parameters are almost identical to those found in rat liver microsomes (K_M 8.90 μM for 1-O-pyrenedecylglycerol, 2.60 μM for tetrahydrobiopterin (13)). 1,10-Phenanthroline, an iron chelator, inhibited alkylglycerol monooxygenase activity generated in CHO cells by transfection in micromolar concentrations (50% inhibition at 1.39 ± 0.38 μM) in a manner similar to observations with rat liver microsomes (19). Presence of tetrahydrobiopterin was only required in the assay mixture, but not for stabilization of the alkylglycerol monooxygenase protein in cells, since addition of sepiapterin to CHO cells did not alter the activity generated upon transfection (Fig. S6*A*).

Injection of TMEM195 and ALDH3A2 cRNAs to *Xenopus laevis* Oocytes. To confirm alkylglycerol monooxygenase sequence assignment to TMEM195 in an independent system, we injected polyadenylated and capped TMEM195 and/or ALDH3A2 cRNA or water into *Xenopus laevis* oocytes, harvested them after 3–4 d and analyzed them for alkylglycerol monooxygenase (13) and fatty aldehyde dehydrogenase (20) activities. We identified tetrahydrobiopterin-dependent alkylglycerol monooxygenase activity in oocytes injected with TMEM195 ($p < 0.05$) which was stimulated about

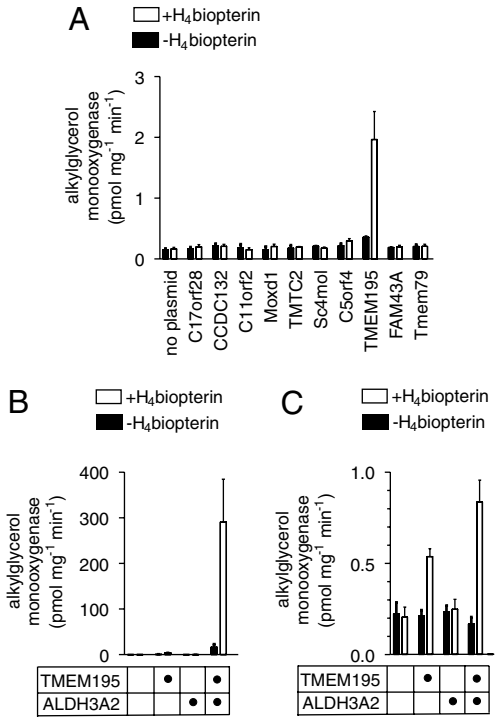

Fig. 2. Alkylglycerol monooxygenase activity generated by nucleic acids. (*A*) Transfection of CHO cells with single expression plasmids. 80,000 CHO cells were transfected with 1 μg each of the indicated mammalian expression plasmids and cultured in presence of 1 μM of the tetrahydrobiopterin precursor sepiapterin for 24 h. Alkylglycerol monooxygenase activity was quantified by incubation with a 1-O-pyrenedecyl-*sn*-glycerol and analysis of pyrenedecanoic acid by HPLC with fluorescence detection (13). Mean values of four measurements of three independent transfections ± SEM are shown for measurements with tetrahydrobiopterin (H_4biopterin), values without tetrahydrobiopterin were determined in duplicate from two independent transfections. (*B*) Cotransfection of CHO cells with TMEM195 and ALDH3A2. Experiments were performed as in (*A*), pairs of plasmids were supplied as 1 μg each. Mean values ± SEM of 3–10 independent transfections are shown, each measurement was done with and without addition of tetrahydrobiopterin (H_4biopterin). (*C*) Injection of *Xenopus laevis* oocytes with TMEM195 and ALDH3A2 cRNAs. 27–69 ng of capped, polyadenylated cRNA prepared from expression plasmids were injected into defolliculated *Xenopus laevis* oocytes. After 3–4 d in culture, oocytes were homogenized and alkylglycerol monooxygenase activity quantified by incubation with a pyrene-labeled alkylglycerol and analysis of pyrenedecanoic acid by HPLC with fluorescence detection (13). Mean values ± SEM of 7–19 measurements on separately injected cRNAs are shown. Each measurement was done with and without addition of tetrahydrobiopterin (H_4biopterin).

twofold ($p < 0.001$) by coinjection with ALDH3A2 (Fig. 2*C*). Like for alkylglycerol monooxygenase activities (Fig. 2 *B*, *C*), fatty aldehyde dehydrogenase activities reached by injection of ALDH3A2 cRNA into oocytes (Fig. S4*B*) were two orders of magnitude lower than those achieved in CHO cells by transfection with an expression plasmid (Fig. S4*A*).

Effect of the Mutation of Conserved Histidines on Alkylglycerol Monooxygenase Activity. Membrane proteins containing the fatty acid hydroxylase motif contain a set of eight conserved histidines, which are all essential for enzymatic activity (21, Fig. S7). We mutated each of these histidines in an expression plasmid for a C terminally 6× myc tagged TMEM195 protein. The nonmutated, tagged alkylglycerol monooxygenase was fully active (Fig. S6*B*). None of the mutated plasmids showed detectable alkylglycerol monooxygenase activity (Fig. S6*B*), although mutated proteins were expressed in an amount comparable to nonmutated wild-type protein (Fig. S6*C*).

Comparison of Alkylglycerol Monooxygenase Activities and Occurrence of TMEM195 mRNA. TMEM195 mRNA levels in mouse tissues and cells available from public databases correlated significantly with alkylglycerol monooxygenase activities measured (Fig. 3*B*). In addition to human cells, mouse, and rat tissues, we detected tetrahydrobiopterin-dependent alkylglycerol monooxygenase activity in chicken liver (strain HB15-FINN, 52.4 ± 1.5 pmol mg^{-1} min^{-1}), zebrafish liver (strain Tübingen longfin, 27.1 ± 5.3 pmol mg^{-1} min^{-1}) but not in *Drosophila melanogaster* (strain Oregon R), *Aspergillus fumigatus* (strain ATCC46645), *Aspergillus nidulans* (strain A89), *Physarum polycephalum* (strain CS310), *Saccharomyces cerevisiae* (strain Y187), or *Escherichia coli* (strain BL21DE3) where all activities were below 1 pmol mg^{-1} min^{-1}. This pattern is consistent with the occurrence of TMEM195 related sequences in the National Center for Biotechnology Information (NCBI) databases, which characterize it to be a gene conserved in Bilateria (Homologene 45620).

Subcellular Localization of a TMEM195—Green Fluorescent Protein Fusion Protein in CHO Cells. Transfection of a plasmid encoding for enhanced green fluorescent protein fused to the C terminus of TMEM195 protein generated alkylglycerol monooxygenase activity in CHO cells (90 pmol mg^{-1} min^{-1}). Live cell confocal microscopy showed a green fluorescence pattern indicating localization in the endoplasmic reticulum (Fig. 4*A*). Costaining of

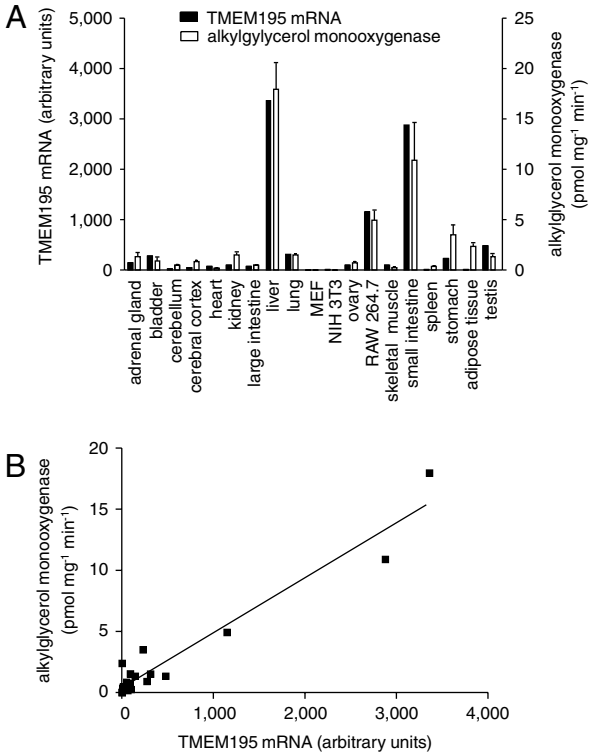

Fig. 3. Alkylglycerol monooxygenase activities and TMEM195 mRNA expression in mouse tissues and cells. (*A*) Distribution of alkylglycerol monooxygenase activities and TMEM195 mRNA expression in mouse tissues and cells. Mouse tissues and cultured cells were homogenized, and alkylglycerol monooxygenase activities determined (13). Mean values of 4–6 independent measurements of tissues ± SEM are shown. Enzyme activities were compared to publicly available gene expression data (BioGPS, http://biogps.gnf.org/). MEF stands for mouse embryonic fibroblasts. (*B*) Correlation of alkylglycerol monooxygenase activities and TMEM195 mRNA expression in mouse tissues and cells. For every tissue and cell shown in (*A*), the alkylglycerol monooxygenase activity measured was plotted against TMEM195 mRNA expression data from BioGPS (http://biogps.gnf.org). Both parameters were significantly correlated (linear correlation, $r^2 = 0.93$, $p < 0.0001$; Spearman rank correlation, $r = 0.75$, $p = 0.0002$).

the plasma membranes (blue, Fig. 4*B*) and of mitochondria (red, Fig. 4*C*) gave no indication for an additional localization in mitochondria or vesicles generated from the plasma membrane (merge, Fig. 4*D*).

Discussion

Sequence Assignment of Alkylglycerol Monooxygenase Activity to Transmembrane Protein 195. Like other researchers in the last 45 yr, we were not able to purify alkylglycerol monooxygenase although we used a robust and sensitive assay for this enzyme activity (13). We could also not assign the sequence by transfection of pools of expression plasmids and rounds of selection until a single clone was obtained, a method that had been successfully used by other researchers to clone genes that could be assayed by a sensitive functional assay, but had not been accessible by protein purification (14). The assignment of the transmembrane protein 195 sequence to alkylglycerol monooxygenase activity presented here is therefore based on the induction of enzymatic activity by transfection of expression plasmids of sequences selected from bioinformatic searches or proteomic analysis of partially purified fractions of the enzyme. In CHO cells, an expression plasmid for transmembrane protein 195 induced a tetrahydrobiopterin-dependent alkylglycerol monooxygenase activity that was tenfold higher than in any tested mouse tissue if sufficient fatty aldehyde dehydrogenase activity was supplied by coexpression. The biochemical properties of this induced activity closely resembled the enzyme activity observed in rat liver microsomes in terms of K_M values for substrate and cofactor, and sensitivity to inhibition by the iron chelator 1,10-phenanthroline.

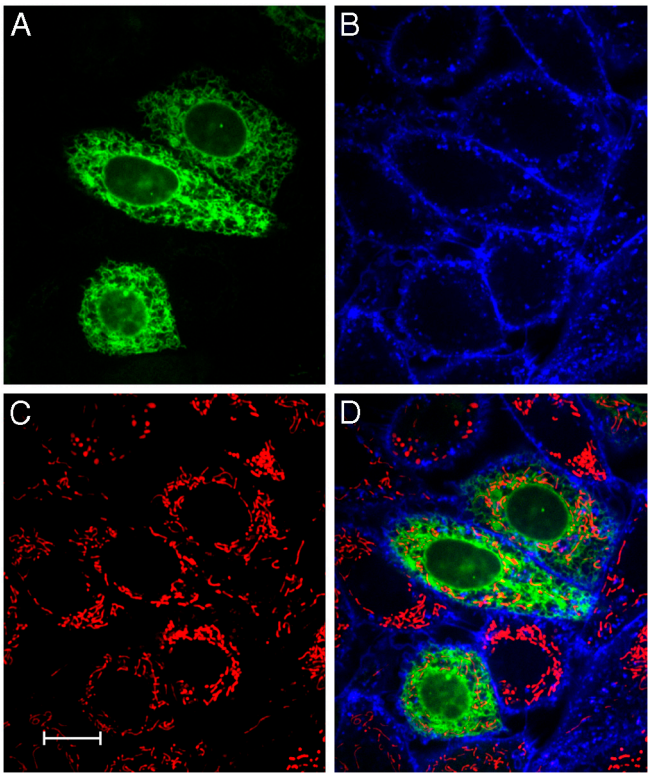

Fig. 4. Live cell confocal fluorescent microscopy of CHO cells transfected with a TMEM195 green fluorescent protein fusion protein. (*A*) Green fluorescence showing the localization of transfected TMEM195 green fluorescent protein fusion protein. The pattern indicates localization in the endoplasmic reticulum. (*B*) Plasma membrane stained in blue with a wheat germ agglutinin conjugate. (*C*) Mitochondria stained in *red* with tetramethyl rhodamine methyl ester. (*D*) Merge, indicating localization of TMEM195 in the endoplasmic reticulum, but not in mitochondria, and not in vesicles originating from the plasma membrane. The white scale bar indicates a distance of 10 μm.

This sequence assignment was confirmed by generation of alkylglycerol monooxygenase activity by injection of transmembrane protein 195 cRNA into *Xenopus* oocytes. In addition, the occurrence of the transmembrane protein 195 and alkylglycerol monooxygenase activity among species (the bilateral animals) is consistent with this assignment. In mouse tissues and cells, alkylglycerol monooxygenase activity correlates well with the amount of transmembrane protein 195 mRNA observed. Localization of recombinantly expressed TMEM195 to the endoplasmic reticulum, the major factory of the cell for producing almost all of its lipids, is consistent with a prominent role of TMEM195 in lipid metabolism and with the observed occurrence of alkylglycerol monooxygenase activity in the microsomal fraction of rat liver (10).

Transmembrane Protein 195 Is a Tetrahydrobiopterin-Dependent Fatty Acid Hydroxylase Type Enzyme. In addition to defining a functional role for transmembrane protein 195, another unique finding of our work is the requirement of tetrahydrobiopterin for a fatty acid hydroxylase motif containing enzyme. While the PFAM04116 fatty acid hydroxylase motif defines an abstract amino acid matrix, previous work has described this motif as a pattern of eight conserved histidines, which are all required for enzymatic activity (21). This pattern is also found in transmembrane protein 195 (Fig. S7), and all of these residues are required for alkylglycerol monooxygenase activity (Fig. S6*B*). Some of these histidines bind the iron atoms constituting the diiron center which is essential for catalysis (22). This feature corresponds well to our observation that alkylglycerol monooxygenase activity can be inhibited by the iron chelator 1,10-phenanthroline (19).

No fatty acid hydroxylase motif containing membrane protein has been reported to be purified to homogeneity from a mammalian source so far. Presumably for all members of this family purification attempts faced similar problems of low stability as we and others experienced with alkylglycerol monooxygenase. Most of the respective human genes, i.e., fatty acid 2-hydroxylase (23), sterol-C5-desaturase (24), and sterol-C4-methyl oxidase-like (25), have been assigned by homology to a characterized yeast gene of similar function. Cholesterol 25-hydroxylase, in contrast, has been cloned by transfection of cDNA pools (26). The radiometric assay for cholesterol 25-hydroxylase used in ref. 26 was sensitive enough to detect the activity upon transfection of pools of 3,000–4,000 clones, whereas we detected only background using pools of 2,500 clones each using our fluorescence-HPLC assay for alkylglycerol monooxygenase (13). In retrospect we speculate that we might have been successful if we had cotransfected ALDH3A2 with the plasmid pools, which led to a 100-fold higher readout in activity (Fig 2*B*).

It will be fascinating to learn why transmembrane protein 195 essentially requires the additional cofactor tetrahydrobiopterin for cleavage of the O-alkyl ether bond which the other fatty acid hydroxylase motif containing proteins apparently do not need. On the other hand, all characterized diiron hydroxylases include a multisubunit hydroxylase, electron transfer proteins, and a cofactorless effector protein that is unique to the diiron hydroxylase family (27). In transmembrane protein 195, tetrahydrobiopterin might substitute for one or more of these additional protein components. TMEM195 may be the only human protein displaying alkylglycerol monooxygenase activity since no obvious sequence homologues were found in the human proteome. Future investigations of alkylglycerol monooxygenase activity of cultured cells with knock down of TMEM195 mRNA will resolve this question experimentally.

Sequence Homology Analysis Suggests That Alkylglycerol Monooxygenase Forms a Distinct Third Group Among Tetrahydrobiopterin-Dependent Enzymes. After characterization of aromatic amino acid hydroxylases, the first known tetrahydrobiopterin-dependent

enzymes, the sequence of another class of tetrahydrobiopterin-dependent enzymes, the nitric oxide synthases, was described about two decades ago (28). Biochemical research has subsequently outlined how nitric oxide synthases differ from cytochrome P450 monooxygenases, making them dependent on the additional cofactor tetrahydrobiopterin and enabling them to avoid formation of an autoinhibitory heme-NO complex (5). Sequence homology suggests that transmembrane protein 195 is equipped with a diiron center for hydroxylation of aliphatic hydrocarbons. This feature distinguishes alkylglycerol monooxygenase biochemically from the other two known classes of tetrahydrobiopterin-dependent enzymes, which contain a heme iron like the nitric oxide synthases or a single, nonheme iron like aromatic amino acid hydroxylases. Similar to this classification by the form of iron contained in the enzymes, primary amino acid sequence comparison clustered tetrahydrobiopterin-dependent enzymes to three independent groups, the aromatic amino acid hydroxylases, the alkylglycerol monooxygenase, and the nitric oxide synthases. These clusters show no primary sequence homology across groups (Fig. 1*B* and Table S2).

The Sequence Assignment will Facilitate the Study of the Physiological Role of Alkylglycerol Monooxygenase. 1-O-Alkylglycerol-derived lipids can modulate signal transduction (30) and are required for nerve and sperm development as well as for protection of the eye from cataract, constitute a component of the GPI anchor, or, as in the case of platelet-activating factor, are mediators themselves (31). Decreased concentrations of ether lipids have been reported to be associated with hypertension (32). The assignment of the sequence of alkylglycerol monooxygenase to transmembrane protein 195 will enable research on the physiological significance of this enzyme. Alkylglycerol monooxygenase is the only enzyme known to degrade the ether bond of lipids without known restrictions to their subclass (10), and may contribute to regulation of the in vivo concentration of these lipids.

Materials and Methods

Materials and methods are described briefly here, a more detailed description is listed in *SI Text*.

Materials. 1-O-pyrenedecyl-*sn*-glycerol was synthesized from glycerol and pyrenedecanol which was obtained from pyrenedecanoid acid by Vitride reduction as described in ref. 13. Recombinant rat fatty aldehyde dehydrogenase was obtained by *E. coli* expression of a Strep-tagged open reading frame obtained by PCR from a rat liver cDNA, and affinity purification (20). Recombinant *P. polycephalum* dihydropteridine reductase was expressed untagged in *E. coli* and purified as described in ref. 33. Pyrenedecanal was obtained from Ramidus AB, Lund, Sweden, pteridines were from Schircks.

Methods. ***Determination of alkylglycerol monooxygenase activity.*** We used a coupled assay: 1-O-pyrenedecyl-*sn*-glycerol is first cleaved by alkylglycerol monooxygenase to pyrenedecanal which is then converted by fatty aldehyde dehydrogenase to pyrenedecanoic acid. Pyrenedecanoic acid can be sensitively monitored by HPLC and fluorescence detection with a detection limit of 1 nmol/L (13). Protein extracts were incubated with 1-O-pyrenedecyl-*sn*-glycerol, tetrahydrobiopterin, dihydropteridine reductase, catalase, fatty aldehyde dehydrogenase, NAD^+, and NADPH for 60 min at 37 °C in 10 μL, the reaction stopped by addition of 30 μL methanol and the resulting mixture analyzed for pyrenedecanoic acid by HPLC. In some experiments of CHO cells transfected with transmembrane protein 195 fatty aldehyde dehydrogenase was supplied by cotransfection.

Determination of fatty aldehyde dehydrogenase activity. Protein extracts were incubated with pyrenedecanal and NAD^+ for 10–20 min at 37 °C in 10 μL, the reaction stopped by addition of 30 μL methanol, and the resulting pyrenedecanoic acid determined by HPLC with fluorescence detection as described in ref. 20.

Proteomic analysis of partially purified alkylglycerol monooxygenase fractions. After solubilization of rat liver microsomes with 0.5% (w/v) cholate and 20% (v/v) glycerol, alkylglycerol monooxygenase was purified over ω-aminohexyl sepharose and hydroxylapatite columns. Proteins differing in the inactive flowthrough and the active fractions eluting from the hydroxylapatite column were determined by two-dimensional gel electrophoresis using combined separation of proteins labeled with different fluorescent dyes, and monitoring of protein concentration differences by three color fluorescence imaging (DIGE system, GE Healthcare). Spots were collected with a spot picker, digested with trypsin in the gel pieces, separated by nano HPLC, and analyzed by electron spray ionization mass spectrometry as described in ref. 34.

Screening of pools of a rat liver expression library. A Superscript rat liver expression library (Invitrogen) was divided to 196 pools containing about 2,500 independent clones. The DNA of the pools was transfected to CHO cells. The cells were cultivated in presence of 1 μM sepiapterin and harvested after 24 h and alkylglycerol monooxygenase activity was measured in protein extracts.

Transfection of CHO cells with candidate genes. Expression plasmids of candidate genes were obtained from Imagenes or Origene, except for ALDH3A2 which was cloned from a rat liver cDNA to a pcDNA3.1+ expression vector (Invitrogen) by standard techniques. Plasmids were transfected to CHO cells using ExGen 500 (Fermentas), the cells cultivated for 24 h in presence of 1 μM of the tetrahydrobiopterin precursor sepiapterin (Schircks), harvested, and alkylglycerol monooxygenase and fatty aldehyde dehydrogenase activities determined.

Mutation of conserved histidines in an expression plasmid of a human TMEM195 protein carrying a C-terminal 6× myc tag. Human TMEM195 was cloned into pEXPR-IBA-103 (IBA), and the C-terminal Strep Tag replaced by a 6× myc tag using standard cloning and PCR techniques. Conserved histidines were then mutated to alanine using the Quikchange procedure (Stratagene), and all mutations were confirmed by DNA sequencing. Plasmids were expressed in CHO-K1 cells together with ALDH3A2, and alkylglycerol monooxygenase activity determined as described above. In parallel, membrane fractions of the cells were prepared and the amount recombinant proteins formed quantified by Western blot using an anti-myc antibody (Santa Cruz Biotechnology) and the ECL plus detection system (GE Healthcare).

Injection of cRNAS to Xenopus laevis oocytes. Capped and polyadenylated cRNAs were generated from the expression plasmids by standard protocols and injected to defolliculated *Xenopus laevis* oocytes with a micromanipulator. After 3–4 d, 2 oocytes each were pooled, homogenized, and analyzed for alkylglycerol monooxygenase and fatty aldehyde dehydrogenase activities.

Subcellular localization of a TMEM195-green fluorescent protein fusion protein in CHO cells. Human TMEM195 was cloned into pEGFP-N1 (Clontech) to generate a protein fused on its C terminus to enhanced green fluorescent protein. Cell growth and live confocal imaging were performed in eight-well chambered coverglasses (Nalge Nunc International). CHO cells were transfected as described above, and live cell confocal microscopic images were acquired 36 h after transfection. Mitochondria were stained in red with tetramethyl rhodamine methyl ester (TMRM, Sigma-Aldrich) and the plasma membrane was stained in blue with an Alexa Fluor 350 wheat germ agglutinin conjugate (WGA, Molecular Probes).

Selection of alkylglycerol monooxygenase candidate genes. The alignment of the tetrahydrobiopterin binding region of aromatic amino acid hydroxylases and nitric oxide synthase presented by Cho et al. (16) was translated to the following protein motif: (R,K)(G,A,L,M,I,V,N)X{1,2}(C,S,T)X{3,4}(A,I,V,L,M)X{4,5}PX{2,3}(S,T)XXPX{2,3}HX{,1}(D,E)(A,M,L,V,I,F)(A,L,M,V,I,F,Y), and Uniprot release 4.6 (1.8 million sequences) searched using the findpatterns program of the gcg program package (version 10.3, Accelrys) locally installed on a Silicon Graphics Origin 2000 server. Proteins were screened for structural features common with phenylalanine hydroxylase using a method for prediction of protein compactness and local structural features (17). PFAM motives were browsed on the public server of the Wellcome Trust Sanger Institute, Hinxton, United Kingdom (http://pfam.sanger.ac.uk/).

Statistics, presentation of data. All computations were performed by the Graph Pad Prism Program 3.0 (GraphPad Software Inc.). Data are presented as mean ± standard error of the mean (SEM) for three independent experiments unless stated differently.

ACKNOWLEDGMENTS. We thank Petra Loitzl, Nina Madl, Rita Holzknecht, and Tamara Pfeiffenberger (Innsbruck Medical University) for expert technical assistance, Igor Baburin and Steffen Hering (University of Vienna) for providing *Xenopus* oocytes, Mohammed Saeed and Reinhard Sigl (Innsbruck Medical University) for providing cell pellets, Helmut Prast (University of Innsbruck), Alexandra Lusser, Johann Schredelseker, and Roswitha Gruber-Sgonc (all Innsbruck Medical University) for providing animal tissues and Hubertus Haas (Innsbruck Medical University) for providing *Aspergillus* material. This work was supported by the Austrian Research Funds "zur Förderung der wissenschaftlichen Forschung" (FWF), projects 19764 and 22406.

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